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14. ABSTRACT The disproportionate incidence and mortality of prostate cancer (CaP) among African Americans (AA) in comparison to Caucasian American (CA) are not well understood. It is believed that high circulating androgens reported in AA men may account for such racial disparities. It has been shown that metastatic tumors maintain functional androgen receptor signaling, suggesting that local (*intracrine*) androgens may contribute to the outgrowth of 'castration-adapted' tumors under androgen deprivation therapy (ADT). Evidence exists for direct correlation between increased obesity and body-mass-index (BMI), which is significantly higher in AA-men, and the risk for aggressive CaP. Active steroidogenic pathways are active in adipocytes and adipose-derived mesenchymal stem cells (ASCs) are often recruited to tumor-stroma. *Our goal will be to exploit the tumor-tropism of normal ASCs to deliver androgen inactivating genes to tumor microenvironments and enable an effective treatment strategy against CRPC.* This will be achieved by: (a) investigate if "intracrine" production of testosterone by osteotropic ASC^{AA} modulates growth and metastatic potential of CaP cells under ADT in vitro and in vivo; (b) determine if α-HSD-expressing osteotropic ASC^{Cont} will nullify the ADMSCAA-mediated CaP cell growth and metastasis in vitro; and (c) examine the efficacy of therapeutically engineered ASC^{Cont} to target and inhibit CaP tumor growth under CRPC *in vivo*. The proposed work will be *innovative*, because it capitalizes on an adjuvant approach for ADT by tumor-site specific inactivation of androgens. Considering the aggressive nature CaP, the outcome of our study is expected to have a positive impact on establishing preventive and/or therapeutic intervention strategies to reduce or circumvent PC, especially among AA-men.

15. SUBJECT TERMS
Prostate cancer, health disparity, stem cells, hormone inactivating enzymes, CRPC, steroidogenic enzymes, African Americans.

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Introduction:

Race, a Risk Factor for Prostate Cancer (CaP). In the US, African Americans have the highest annual incidence of CaP, at 272 new cases per 100,000 men [1]. In 2009, 230,000 men were diagnosed with CaP, and more than 38,000 afflicted men are expected to die [2]. Although the etiology remains largely unknown, racial make-up has been identified as one of several risk factors for CaP. African-American (AA) men bear a disproportionately heavy burden from this disease with incidence and mortality rates over 50% higher than Caucasian American (CA) men [3, 4]. Furthermore, AA men are more likely to develop CaP at an earlier age, have higher rate of Gleason-7, aggressive tumors, and metastasis, and exhibit a poorer survival rate than CA males [3-7]. Socioeconomic and environmental factors, such as diet, access to care, and screening, have been cited as factors contributing to the more clinically aggressive CaP in AA patients [8, 9]. Family history accounts for 5-10% of total CaP cases [8, 9], and it does not differ among AA, Asian Americans, and CA men [10, 11]. A more biologically aggressive CaP has been proposed as one possible explanation for the younger age at presentation and disease progression in AA men compared with CA men [12, 13].

Obesity and BMI as Risk Factors for CaP progression in AA men. Prostate cancer incidence and mortality rates correlate well with the average intake of fats, including polyunsaturated fats [14]. *In vivo* and *in vitro* models have demonstrated a decreased rate of proliferation of prostate tumors with reduced fat intake [15-17]. One meta-analysis showed a 5% excess risk of developing prostate cancer for each 5 kg/m^2 increment of BMI [18]. When disease stage was considered, the analysis showed a rate ratio for advanced cancer of 1.12 per 5 kg/m^2 increment. An analysis from the CaP Prevention Trial noted that compared with men with a BMI below 25 kg/m^2, those with a BMI above 30 kg/m^2 had an 18% decrease in the risk of low-grade cancer, but a 29% increase in the risk of high-grade cancer [19]. In addition, obesity has been linked to aggressive CaP [20] and increased BMI has also shown a positive correlation to Gleason score and positive surgical margins [21]. The latter is critical as it can be an indicator for disease relapse. However, the mechanisms linking obesity to CaP development and progression are not fully understood. Since the prevalence of obesity is significantly high in AA men [22], accounting for 37.3%, unraveling such mechanisms is of paramount significance.

"Intracrine androgens" and CaP progression. Androgen-deprivation therapy (ADT) has been the mainstay treatment for patients with metastatic CaP [23]. Although initially effective, hormonal therapy is marked by progression to castration-resistant prostate cancer (CRPC) over a period of 18–20 months, with median survival of 1–2 years. Importantly, large body of evidence indicate that in the setting of 'castrate' serum testosterone levels, prostatic androgen concentrations remain at approximately 10–25% of the levels found in untreated patients [24-26] well within the range capable of mediating continued androgen-receptor (AR) signaling and gene expression [27]. Moreover, residual intra-prostatic androgens are implicated in nearly every mechanism whereby AR-mediated signaling leads to the development of castration-resistant disease [28]. The increased expression of androgen-metabolizing genes within castration-resistant metastatic tumors [29] strongly suggests that up-regulated activity of endogenous steroidogenic pathways is driving the outgrowth of 'castration-adapted' tumors. The source of residual androgens within the prostate tumors of castrate men has not been fully elucidated, but has been attributed to the uptake and conversion of circulating adrenal androgens [30]. Whether the de novo biosynthesis of androgens from cholesterol or earlier precursors occurs within castration-resistant metastases is not known [28] but has significant implications for treatment strategies targeting sources of androgens exogenous to the prostate versus 'intracrine' sources active within the actual metastatic tumor microenvironments.

ACCOMPLISHMENTS:

What were the major goals of the project?

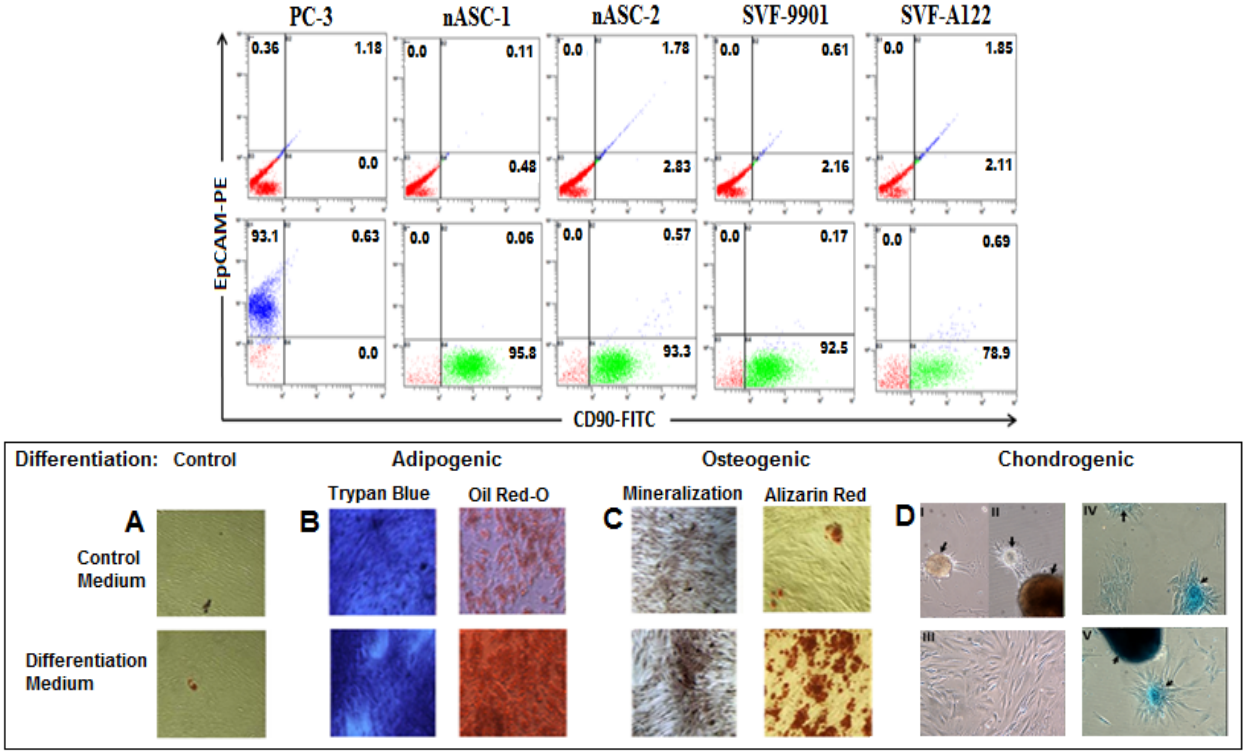
SPECIFIC AIM -1: ***Investigate if "intracrine" production of testosterone by enriched ADMSC^{AA} modulates growth and metastatic potential of CaP cells under ADT in vitro and in vivo.***

Task-1. Generate a large number of well characterized ADMSC stocks from multiple donors. 1-6

1.1. Obtain lipoaspirates from normal donors and CA and AA CaP-patients and isolate and culture ADMSCs by using protocols already established in our laboratories.

More than 50 ADMSC isolates were prepared from both populations. Fresh periprostatic fat tissue (approximately 1 gm) procured from AA and CA men with PC was washed three times in PBS and minced on ice to approximately 1 mm^3 pieces as we previously described. The minced tissue was then suspended in 2 mg/ml of collagenase type-I (GIBCO, Invitrogen, Carlsbad, CA) constituted with PBS and subsequently incubated at 37°C in a shaking water bath for 2-2.5 hr. The cell suspension was then filtered through 70 µm and 40 µm cell strainer respectively (BD Biosciences, MD) to remove tissue debris. Mature adipocytes were removed by centrifugation (1,500x*g* for 10 min) followed by another wash in PBS. The resulting stromal vascular fraction (SVF) pellet is suspended and incubated for 2 min in red blood cell lysis solution (0.15 M ammonium chloride, 10mM potassium bicarbonate and 0.1 mM EDTA). Stem cells were washed in 2 ml 1% BSA (Sigma-Aldrich, MO), suspended in DMEM/F12 medium (1:1; v/v) supplemented with 10% FBS and 1% antibiotics-antimycotic solution (penicillin G, streptomycin and amphotericin B; Mediatech, Herndon, VA) and maintained at 37^{O}C with 5% CO_2. At 70% confluence, the normal ADMSCs and patient derived ADMSCs were cultured in phenol red free DMEM/F12, supplemented with 10% charcoal-stripped FBS for 24 hr. The CM was collected, filtered (0.2 µM) and stored at -20^{O}C until used.

1.2. Analysis of stem cell characteristics in ADMSCs by flow cytometry and lineage-specific differentiation, and development of large batches of liquid-N_2 stocks for future experiments.



The purity and characteristics of isolated ADMSCs were determined by FACS analysis (upper panel, previous page) and panel and by adipogenic, osteogenic and chondrogenic differentiation media (lower panel; previous page).

<u>Task-2</u>. Compare the *in vitro* effects of CaP cells on androgen production by ADMSCs and determine the effects of ADMSC cocultures on CaP cell growth.

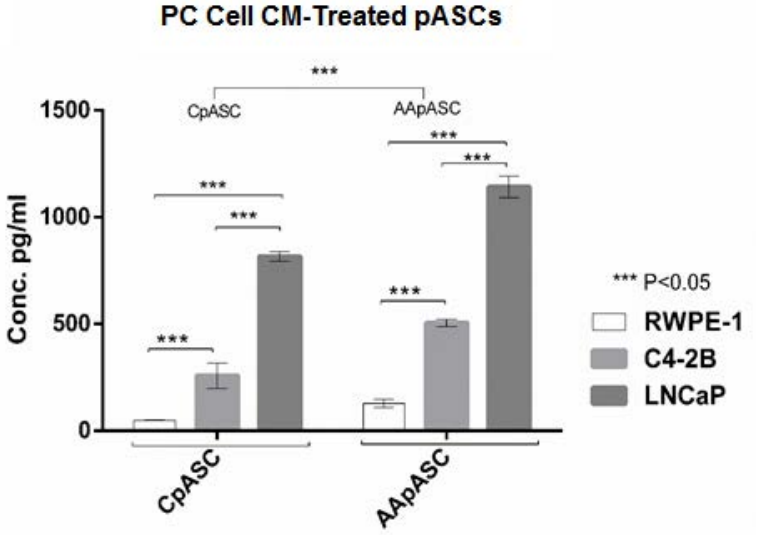
2.1. Harvest and freeze aliquots of conditioned medium (CM) from different CaP cell lines, e.g. LNCaP, C4-2B, PC-3, MDA-PCa-2a and MDA PCa-2b cell lines.

We have prepared condition media (CM) from different prostate cancer cell lines. Briefly, cells were cultured in complete media until ~ 70% confluency. After washing (3x in BPS), cells were grown in serum-free media for 24 hrs. After centrifugation (1,000xg, 10 min) and filtration (0.2 µM), the CM were frozen at -20^{o}C until used.

2.2. Expose ADMSC^{Cont}, ADMSC^{CA} and ADMSC^{AA} cells to CM from the above CaP cells and measure testosterone (T) and dihydrotestosterone (DHT) levels in culture media by ELISA assays.

The nonmalignant, immortalized prostate cells (RWPE-1) and androgen-dependent LNCaP cells were purchased from ATCC (Manassas, VA). The isogenic CRPC C42-B cell line was a generous gift from Dr. L.W. Chung (Cedars-Sinai, Los Angeles, CA). LNCaP, C4-2B and PC-3 cells were cultured in RPMI-1640 medium (ATCC) supplemented with heat-inactivated 10% FBS and 1% penicillin/streptomycin (Invitrogen Life Technologies, MD). RWPE-1 was maintained in keratinocyte serum free media supplemented with EGF and bovine pituitary extract (Invitrogen Life Technologies, MD). For preparation of CM, both normal (RWPE-1) and PC cells were cultured up to 80% confluence in appropriate media following which they were washed thrice with DPBS and cultured in serum free, phenol red free and 1% penicillin/streptomycin for 24 hours. The CM was then collected and centrifuged at 1,500 RPM for 10 minutes, filtered (0.2 µm) and stored at -20°C until used.

The ADMSC^{Cont}, ADMSC^{CA} and ADMSC^{AA} were cultured in DMEM/F-12 supplemented with 10% charcoal stripped FBS up to 80% confluence. The ASCs were washed with PBS thrice and were cultured in Control serum- free medium, CM (1:1; v/v) or MVs (5-10 µg/mL) was prepared derived from RWPE1 or PC cells. The ASCs were then harvested at different time points (up to 96 hours) and the expression of AMEs (SRD5A1, SRD5A2, and AKR1C3 (as known as 17bHSD5), 17bHSD3, HSD17B1, HSD17B2, HSD17B3 and 3-β-HSD) in ASCs were analyzed by quantitative RT-PCR. Primer sequences were obtained from Getprime database and ordered from IDT (Coralville, IA). A Testosterone EIA was performed as per manufacturer's protocol (Cayman Chemicals, Ann Arbor, MI) to quantitate the release of testosterone. The results were normalized to controls and data was expressed as fold change ± SE from three independent experiments.



To verify the effect of AME upregulation on testosterone concentration *ex vivo*, ADMSC^{CA} (CpASC) and ADMSC^{AA} (AApASC) were treated with CM derived from PC cells or non-tumorigenic cells for 24 hours and DHT concentration was estimated using testosterone ELISA kit. Exposure of patient-derived ADMSCs to LNCaP CM lead to a higher secretion of testosterone compared to the exposure to CM derived

from non- tumorigenic cells and C4-2B cells (right Fig). Importantly, $ADMSC^{AA}$ showed a significantly higher average concentration of testosterone compared to $ADMSC^{CA}$ upon exposure to PC cell-derived CM ($p<0.05$).

2.3. Obtain total RNA and proteins from ADMSCs, control and CaP CM-exposed, and determine the change in expression of different steroidogenic enzymes by qRT-PCR and Western blot.

Control serum-free medium, CM (1:1; *v/v*) or MVs (5-10 µg/mL) derived from RWPE1 or PC cells were added to nASCs or pASCs (70% confluent) pre-cultured in medium supplemented with 10% charcoal-stripped FBS and subsequently harvested at various time frames (up to 96 hr). The expression of AME in ASCs was analyzed by qRT-PCR. The release of androgens in media was measured by an ELISA kit as per manufacturer's recommendations (Cayman Chemicals, Ann Arbor, MI). The sequences of AME amplimer sets used in qRT-PCR analysis are listed in Table 1. The results were normalized to controls and data was expressed as fold change ± SE from three independent experiments.

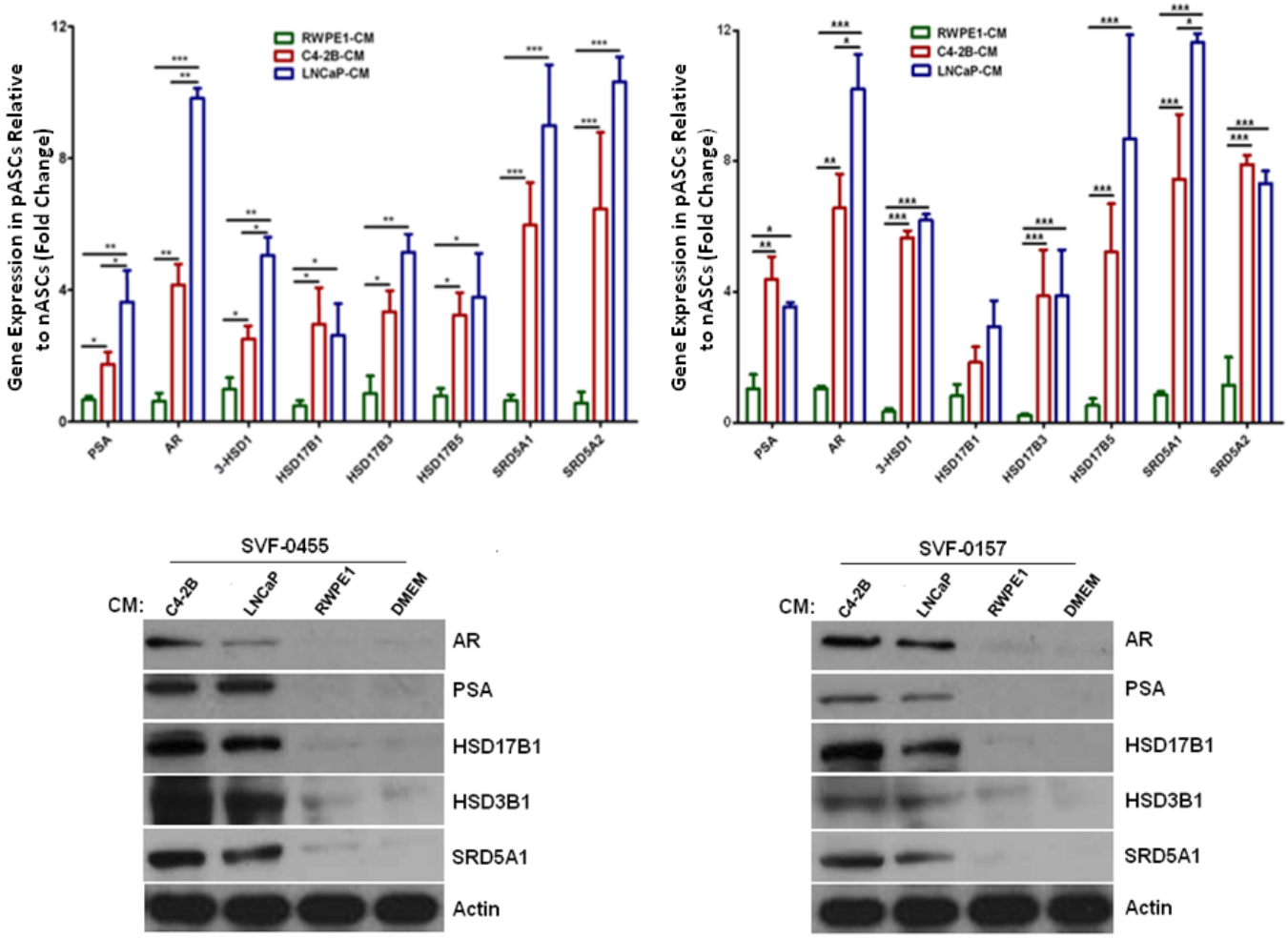


Fig. 1: PC cells confer transcriptional upregulation of AR, PSA and steroidogenic gene expression in pASCs *in vitro.* Real-time PCR analysis of gene expression of AR, PSA, AME in pASCs cells, designated SVF0157 **(a)** or SVF0455 **(b),** incubated with CM derived from C4-2B, LNCaP or RWPE1 cells for 24 hrs. Prostate specific markers and AMEs were significantly upregulated in pASCs exposed to CM from C4-2B or LNCaP cells, as opposed CM from RWPE1 cells. Data are expressed as fold change ± s.e.m. after normalization to nASCs (*n*=3). *, ** and *** denotes significance at p<0.5, p<0.01 and p<0.001, respectively. **c, d**, Immunoblot analysis of AR, PSA and AME protein levels in two representative pASCs (SVF0157 and SVF0455) exposed to growth media alone (DMEM) or to CM (50%) from C4-2B, LNCaP or RWPE1 cells for 24 hr.

While morphology remained unchanged, the condition medium (CM) of LNCaP and CB-2B stimulated the growth of enriched pASCs (SVFA122 and VF-0455) in a concentration dependent manner (*data not shown*). The growth induction was associated with transcriptional upregulation of AR (3 to 6-fold) and PSA (2 to 3.5 fold) by PC cell CM in pASCs, derived from AA men, compared to RWPE1 cell CM or control medium (Figure 2). Next, we examined the expression of androgen metabolizing enzymes (AME) involved in *de novo* biosynthesis of testosterone (T). PC cell CM treated pASCs (SVF0157, SVF0455), but not nASCs, induced gene expression of 3-β-hydroxysteroid dehydrogenase (3-β-HSD) and 17-β-hydroxysteroid dehydrogenase (17beta-HSD) subtype 1, 3 and 5 compared to CM of normal prostate epithelial cells (RWPE1) (Figure 1). Similar expression profile was detected in two additional pASCs from AA men (SVFA122 and SFVB123) (*data not shown*). The expression of AR, prostate specific antigen (PSA), and AME gene expression by pASCs was corroborated by immunoblotting (Figure 2). In comparison to nASCs, an increase in SRD5A1 and 2 transcripts, which encode for 5α-reductase subtype 1 and 2 required for conversion of T into dihydrotestosterone (DHT), was also observed in four pASC isolates from AA men in response CM of PC cells in a time-dependent manner (Figure 3, next page). The induction of steroidogenic genes was coupled by T production by four pASC isolates pretreated with CM of LNCaP in comparison to controls.

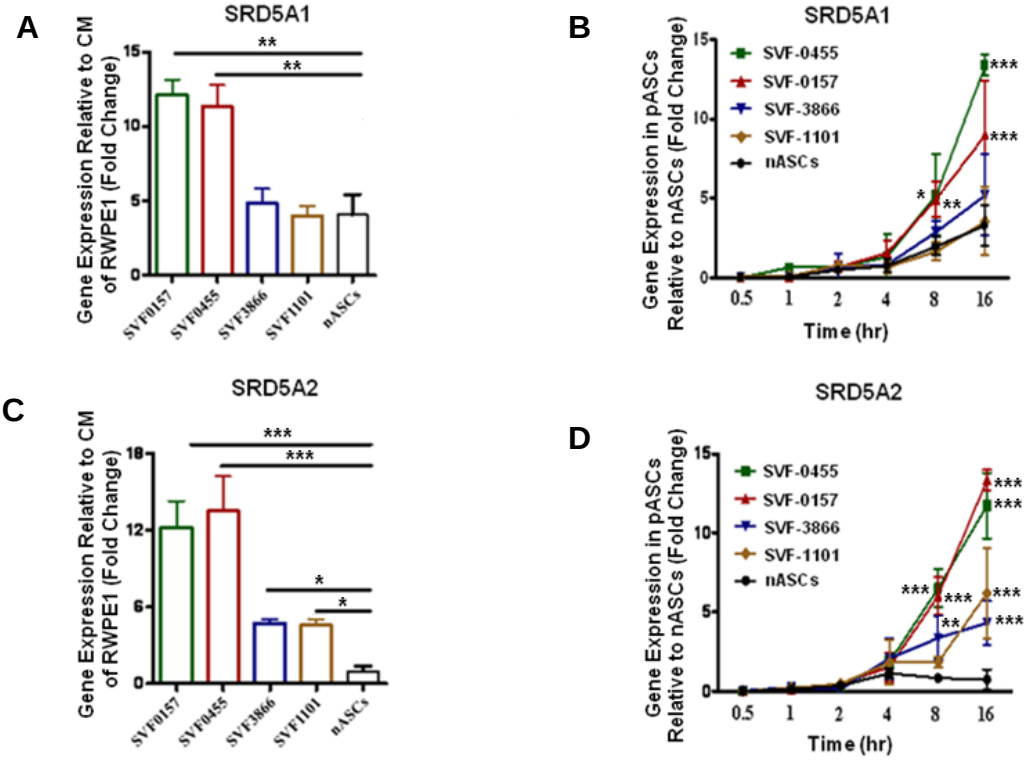


Figure 2: PC cell soluble factors confer transcriptional upregulation of DHT converting enzymes by patient derived ADMSCs (pASCs) *in vitro.* **a**, **b**, Gene expression of DHT converting enzyme 5-alpha-reductase isoforms (SRD5A1 and 2) in four representative pASCs (SVF0157, SVF0455, SVF3866 and SVF1101) exposed to CM derived from PC cells or RWPE1 cells for 24 hr. **c, d**, Temporal expression of SRD5A1 and SRD5A2 genes were monitored in pASCs exposed to CM from PC cells or RWPE1 cells for 0.5 – 16 hrs. Data are expressed as mean ± s.e.m. in triplicate measurements. *, ** and *** denotes significance at p<0.05, p<0.01 and p<0.001 relative to CM of RWPE1 cells or nASCs. (*n*=3).

We also investigated if there are racial differences in expression of AME by ADMSCs derived from African Americans (AA) and Caucasian American (CA) men. ADMSCs were exposed to CM of PC cells as shown above and the total RNA was analyzed for AME expression by qRT-PCR. The results shown in Figure 3 (next page) demonstrated that key AMEs, such as AKR1C3, SDR5A1 and 2 are significantly expressed in ADMSCs derived from AA compared to CA men.

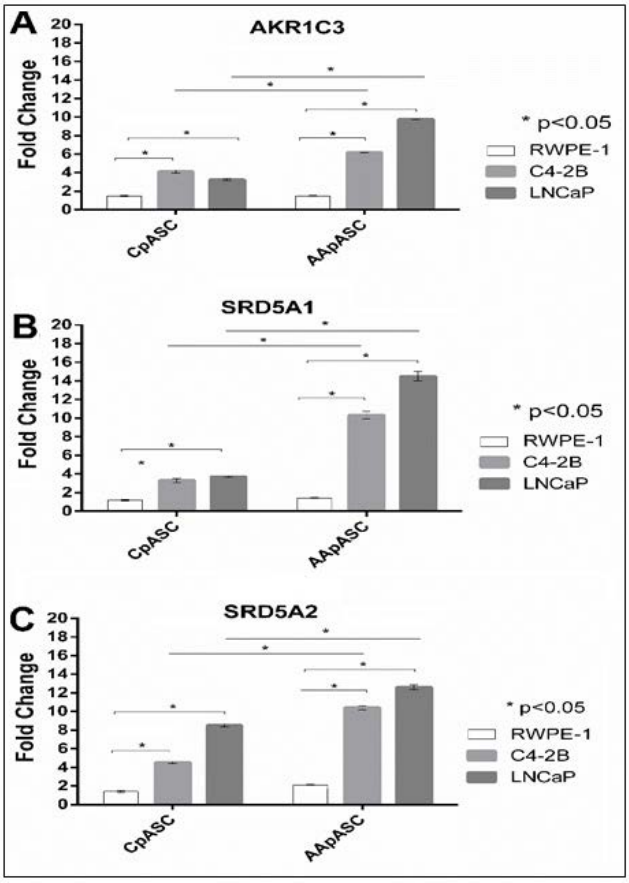
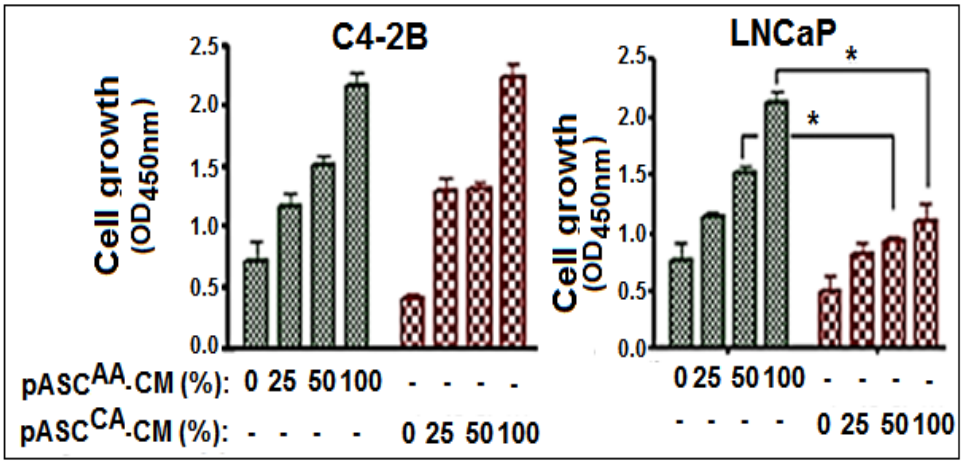


Figure 3. Fold change upregulation of androgen-metabolizing enzyme (AME) genes (AKR1C3, SRD5A1 and SRD5A2) in ADMSCs from AA-men (AApASCs) and Caucasian Americans (CpASCs) upon treatment with PC cell-derived CM. Fold change was calculated using Livak's method. Calculated fold change was compared using non parametric ANOVA (Kruskal Wallis test) followed by post hoc analysis using a Tukey test and significance was determined at $p<0.05$.

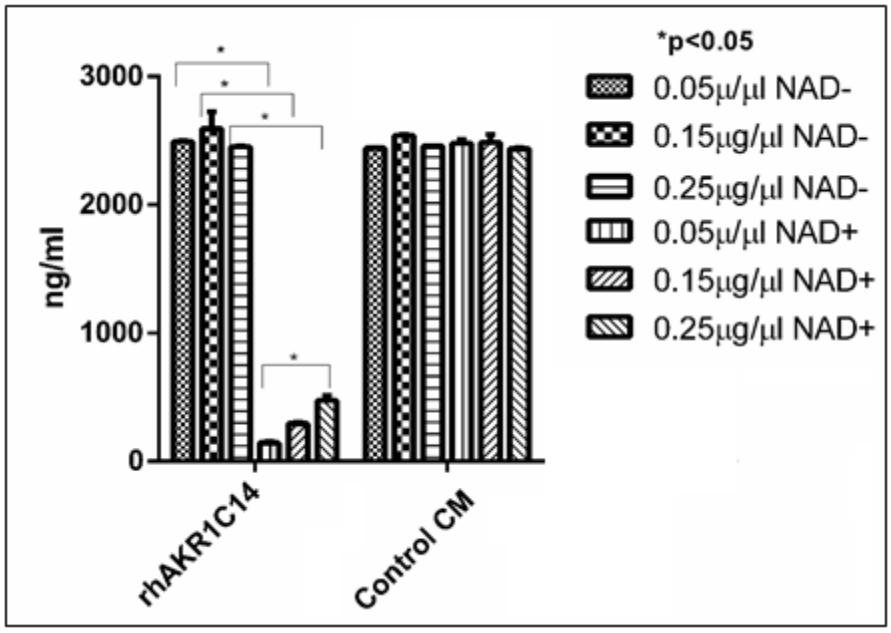
2.3. Expose different CaP cells, growing in either complete or charcoal stripped media, to CM from the above ADMSCs and measure CaP cell growth.

LNCaP or C4-2B cells were grown in charcoal-stripped media and various concentrations of CM of ADMSCs pre-stimulated with CM of the same PC cells for 48 hrs. As shown in the Fig (right), the CM of ADMSCs primed growth of both PC cell lines in a concentration-dependent manner, with more robust effect observed by $ADMSC^{AA}$ cells



2.4. Coculture ADMSC^{AA} cells with CaP cells (GFP-labeled) and measure percent change in PSA- and/or GFP +ve cells, in the presence or absence of androgen inactivation by recombinant α-HSD.

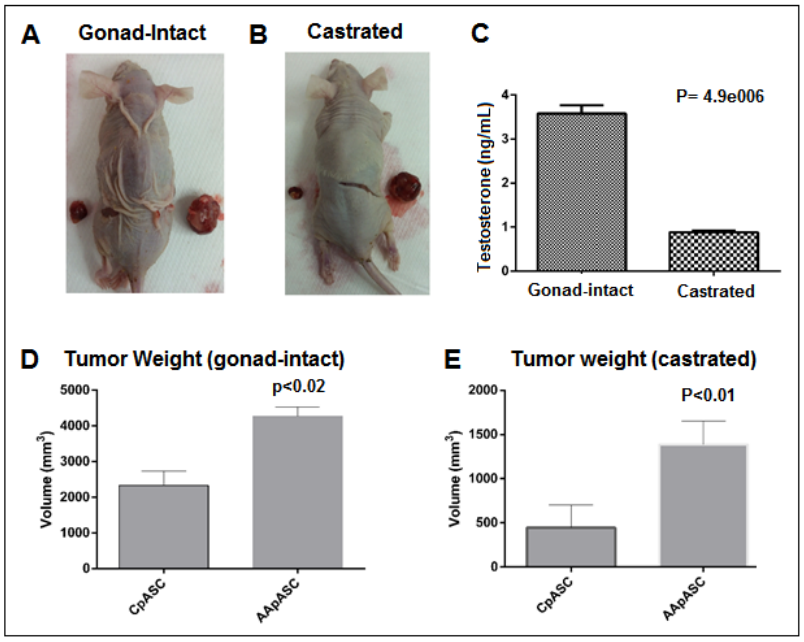
LNCaP cells were grown in CM of ADMSCs pre-stimulated with CM of LNCaP cells as shown above in presence or absence of various concentrations of human recombinant α-HSD or in media supplemented with 10 nM DHT with and without 2.5mM NAD for 48 hrs.. ELISA was performed to measure DHT in the culture media. The results showed significantly lower DHT concentration in LNCaP cells treated with recombinant AKR1C14 compared to treatment with CM from control cells.



<u>Task-3</u>: Investigate the *in vivo* effects of ADMSCs coinjected with CaP cells on tumor burden in nude mice and demonstrate the role of ADMSCs in androgen production and tumor growth.

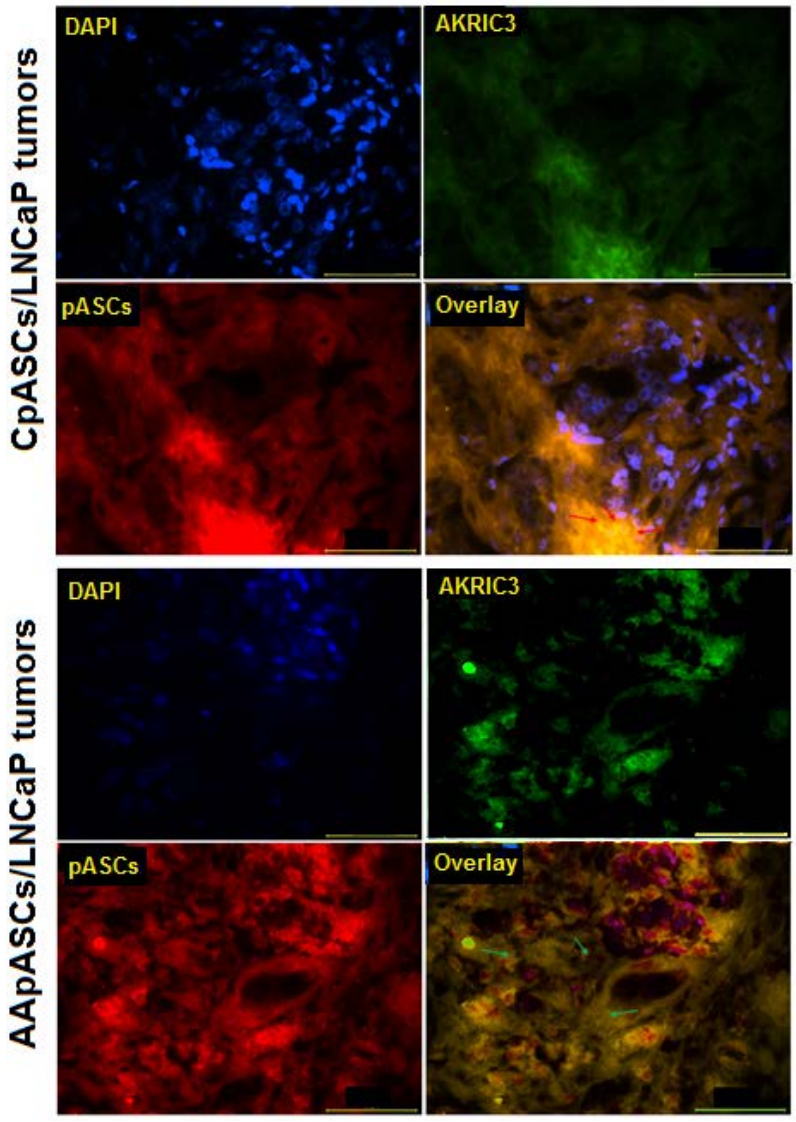
3.1. Monitor tumor size in male nude-mice (gonad intact or castrated) subcutaneously injected with CaP cells, alone and/or with ADMSC^{AA}, at different time points post xenograft.

We demonstrated that ADMSCs derived from African American with PC (ADMSC^{AA}) promote LNCaP cell tumor growth in gonad-intact and castrated nude mice. Shown are representative images of tumor volumes of LNCaP tumor formation co-transplanted with GFP-expressing ADMSC^{AA} (right flank) or those procured from Caucasian Americans (CA) (left flank) in gonad intact (A) and castrated (B) mice. C, depicts serum concentration of testosterone as



estimated by ELISA was significantly lower in castrated mice compared to gonad-intact mice (p value<0.001). LNCaP cells co-inoculated with ADMSC^{AA} showed significantly higher tumor volumes than the LNCaP cells co-inoculated with ADMSCs derived from CA men in both in gonad-intact and castrated mice. LNCaP cells co-inoculated with ADMSC^{AA} showed significantly higher tumor weight than the LNCaP cells co-inoculated with ADMSCs derived from CA men in both in gonad-intact (D) and castrated mice (E). The results suggest that ADMSCs from AA-men possess intrinsic properties that preferentially support tumor growth than their counterparts from CA men.

3.2. In similar *in vivo* studies using labeled CaP cells (ZsGreen) and labeled ADMSCs (mCherry) harvest tumors and determine steroidogenic enzymes in engrafted ADMSCs by IHC and IFM analysis. We demonstrated that the tumor -engrafted ADMSC^{AA} cells express androgen-metabolizing enzymes (AMEs), and presumably androgens, to support growth of the androgen-dependent LNCaP cells in gonad-intactmic. Shown on the figure (right) are representative immunofluorescence images of the AKR1C3 protein expression (red color) in formalin fixed paraffin embedded (FFPE) mouse tumor sections of LNCaP cells co-inoculated with GFP-expressing ADMSCs from CA men (*upper panels*) and LNCaP tumor -engrafted ADMSC^{AA} (*lower panels*) in castrated mice. The pASC engraftment was examined using anti-GFP anti-body shown in red. The expression of AKR1C3 was detected using green-fluorescent Alexa Fluor® 488 secondary antibody. The nuclear stain is DAPI shown in blue (DAPI) and the co-localization of AKR1C3 and stem cells is shown by overlaying (*green arrows*) the images (*yellow color*). Scale bar= 50 µm.



The results support the hypothesis that tumor-tropic ADMSCs from PC patients, especially AA-men, support growth of PC cells through de novo synthesis and production of androgens in the tumor microenvironment. This view was further strengthened by the expression of other key AMEs involved in de novo synthesis and production of androgens by ADMSCs co-inoculated with LNcaP cells (see Fig 11, first manuscript).

Further analysis of CM of PC cells, demonstrate that trafficking of PC cell-derived exosomes into the tumor-tropic ASCs are the culprits for upregulation of AME transcripts and de novo synthesis of androgen by these cells.

SPECIFIC AIM-2:- ***Determine if α-HSD-expressing osteotropic ADMSC^{Cont} will nullify the* ADMSC^{AA}*-mediated CaP cell growth in vitro.*** Based on our preliminary studies, the *working hypothesis* here is that only a selective subset of ADMSC^{Cont} has higher propensity to adhere and transmigrate through human bone marrow endothelial cells (BMEC) towards CaP cells *in vitro*. Osteotrpic ADMSC^{Cont} will be genetically engineered to express α-hydroxysteroid dehydrogenase (α-HSD), a testosterone and DHT inactivating enzyme, and examined for their ability to suppress ADMSC^{AA}-mediated CaP cell growth *in vitro*.

Task-4: Carry out *in vitro* assays to select for those ADMSC^{Cont} cell subpopulations, which possess increased osteotropism and tumor-homing potential (ADMSC^{Sel} cells)-months 9-14

4.1. ***Transmigration experiments in trans-well culture (TWC) chambers containing prostate cancer, with fluorescent-labeled ADMSC^{Cont} on top and CaP CM in bottom.***

4.2. ***Harvest the transmigratory ADMSC subpopulations by FACSorting and grow to large scale to obtain several ADMSC clones that possess higher osteotropism and tumor-homing ability (ADMSC^{Sel} cells).***

The normal adipose tissue derived stem cells (ADMSC^{Cont}**)** were generously provided by Dr. Jeffery M. Gimble (Pennington Biomedical Research Center, Baton Rouge, LA). We have successfully isolated three different populations with tumor-homing potential. Briefly, ADMSC^{Cont} populations with high tropism towards bone metastatic PC cells (LNCaP) were enriched using an *in vitro* trans-endothelial migration (TEM) system. The human bone marrow endothelial cells (hBMEC-1) barrier (kindly provided by Dr. Graça D. Almeida-Porada, University of Nevada, Reno, NV) cultured onto Matrigel™-coated membrane inserts (8 µm pore size) in 12-well plates to generate a confluent hBMEC-1 barrier on the upper chamber. The permeability of the microvessel barrier was checked with Evans blue dye by colorimetric assays. The conditioned medium of LNCaP cells (CM) was added to the lower chamber. The CM was prepared by growing the LNCaP cells in complete medium (RPMI, 10% FBS 7 antibiotics) to 80% confluency. The medium was then removed and replaced with serum-free RPMI medium for 24 hr. The CM is filtered (0.2 µm) and stored in -20^{o}C until used. The ADMSC^{Cont} cells (1 x 10^{5}) were added onto the microvessel barrier and allowed to migrate towards the CM in the lower chamber for 48 hr. Only ASC isolates with tropism towards PC CM (ADMSC^{Sel}) were propagated (passage <3), stored and used in subsequent experiments.

Task-5. Construct a lentiviral vectors (LV) expressing a secretable 3α-HSD enzyme and optimize the LV-transduction and transgene expression in enriched ADMSC^{Sel} cells. Months 14-20

5.1. ***Construct the delivery plasmid, by cloning AKR1C4 cDNA into the expression vector pLVX-IRES-ZsGreen1.***

5.2. ***PCR amplify the PTD-AKR1C9 region and subclone into pFUSE-mFc2 which contains the 21 amino acid secretory signal (ss) from the IL2 gene.***

Figure-1

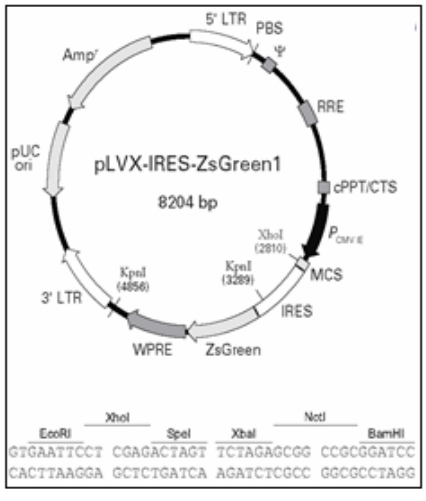
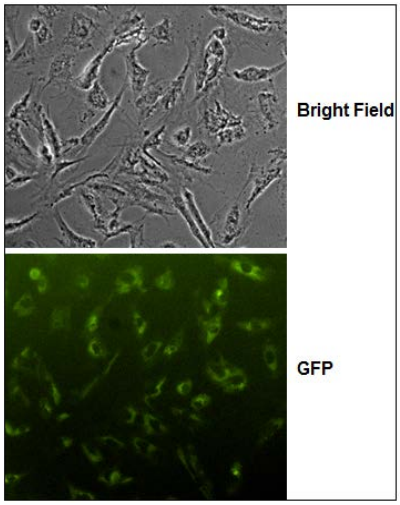


Figure-2



In another set of experiments, we purchased a lentivius construct, pLVX-IRES-ZsGreen1, from Clontech, Inc. **(Fig.1)**. The construct is an HIV-1-based, lentiviral expression vector that allows the simultaneous expression of protein of interest and ZsGreen1 in virtually any mammalian cell type, including stem cells. ZsGreen1 is a human codon-optimized variant of the reef coral *Zoanthus sp*. GFP, ZsGreen. The vector expresses the two proteins from a bicistronic mRNA transcript, allowing ZsGreen1 to be

used as an indicator of transduction efficiency and a marker for selection by flow cytometry. The expression plasmid was used to generate a lentivirus expressing GFP (EF1-alpha GFP lentivirus) at the Applied StemCell, Inc. Briefly, 24 hr before transfection, Lenti-X 293T cells/100 mm plate were cultured to 80–90% confluency. Next, plasmid and Xfect Polymer solutions were mixed and incubated for 10 min at RT and subsequently added to the cultured cells. After 4 hr, the transfection medium was replaced with fresh complete growth medium and cells were incubated at 37°C for an additional 48 hr. The lentiviral supernatants were pooled, centrifuged and filtered through a 0.45 µm filter to remove cellular debris. Virus production was verified with Lenti-X GoStix™. The EF1-alpha GFP lentivirus virus titer was 2.61 ± 0.29 x 10^8. The viral stocks were aliquoted and stored at –80°C.

The ADMSC^{Cont} cells were transduced with pLV-GFP using a standard protocol. Briefly, 10,000 cells/well were plated in a 24-well plate overnight in DMEM-F12 medium. After washing, the medium was replaced in a fresh phenol-red free medium containing 10 µg polybrene and pLV-GFP and cell were cultured overnight. The medium was changed and transduction efficiency was ~ 90%, as assessed with fluorescence microscope **(Fig. 2)**. The GFP-expressing ADMSC^{Cont} cells were further sorted by FACS and the enriched populations were propagated in cultured and stored for future *in vivo* experiments.

5.3. *Isolate the fragment coding for IL2ss-HSD and clone into the polylinker site of pShuttle where transgene is under the control of CMV promoter/enhancer to generate pLVX-IL-2SS-Akr1c14-IRES-GFP.*

5.4. *Remove the CMV enhancer sequences from pEF1-IL2ss-PTD-HSD and insert the Osx binding element (–149 to +27) of human collagen gene (COL11A2) to generate the pOsx-IL2ss-PTD-HSD.*

5.5. *Cotransfect 293 T-cells with the pseudotyping plasmid, packaging plasmid, and transgene plasmid and harvest LV particles.*

The Akr1c14 (NM_138547) rat cDNA clone coding for 3α-hydroxysteroid dehydrogenase (3α-HSD) was obtained from Origene. The 3α-HSD, also known as aldo-keto reductase family 1 member C4, is an enzyme that in humans is encoded by the *AKR1C4* gene. The IL2-SS (signal sequence) was synthesized (IDT) as shown in **Fig. 3**. The IL-2SS enables secretion of Akr1C14 gene production by the ADMSC^{Cont} cells.

Figure-3

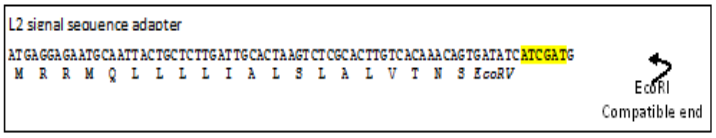


Figure-4

Initially, the Akr1c14 gene was cloned in-frame with IL-2SS at the N-terminus in pCR-II plasmid using EcoRI and EcoRV restriction enzymes and adaptors and the DNA insert was sequence verified. The IL-2SS- Akr1c14 sequence was PCR amplified with SpeI and NotI anchored primers. The PCR product was subcloned in SpeI-NotI digested pLVX-IRES-ZsGreen1 plasmid (pretreated with CIP) to generate pLVX-IL-2SS- Akr1c14-IRES-GFP construct, as shown in **Fig. 4**. The DNA insert was in-frame and was sequenced verified.

We employed the strategy described in Task 4.2 (Applied StemCell, Inc.) to generate lentivirus expressing the pLVX-IL-2SS- Akr1c4-IRES-GFP gene product. The viral titer was 3.26 ± 0.53 x 10^8.

5.6. *Transduced ADMSC^{Sel} cells with the LV particles (10-100 MOI) and monitor transgene expression by RT-PCR and its secretion in the media by ELISA assays.*

For PCR analysis, total RNA was extracted and cDNA synthesis was performed using standard protocols. from stem cells transduced with control plasmid or the transgene-expressing plasmid. Target genes were amplified with β-actin and Akrc14 specific primers and examined for expression using ethidium bromide stained gels.

Figure-5

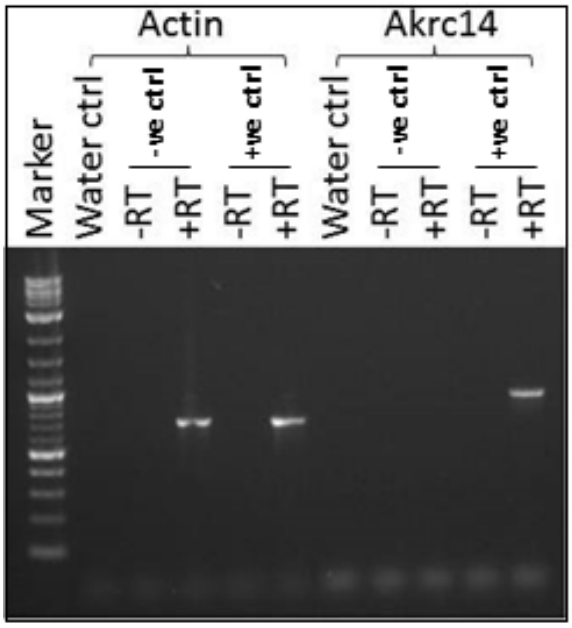
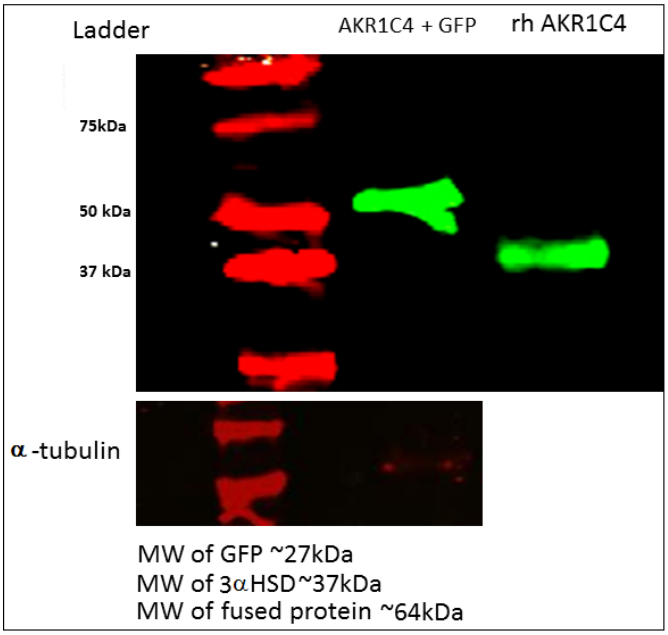


Figure-6



As shown in **Fig. 5**, the transgene (Akr1c4) is expressed in the in stem cells transduced with transgene-expressing pLVX-IL-2SS-Akr1c4-IRES-GFP construct but not by the control or negative controls. The loading controls are shown by expression of actin.

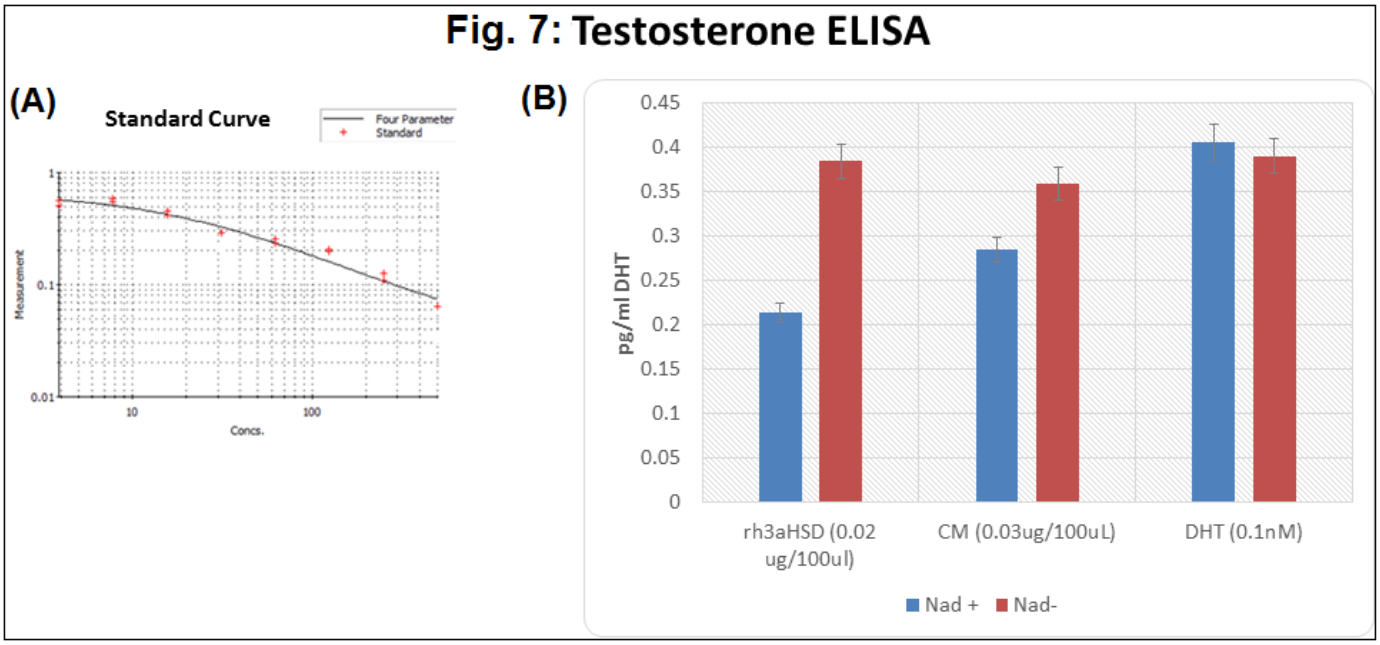
For Western blot analysis, the ADMSC^{Se} were transduced with control plasmid (pLVX-IRES-GFP) or the transgene-expressing pLVX-IL-2SS- Akr1c14-IRES-GFP as shown above and cells were cultured for 48 hr. The medium was collected and examined for transgene expression and release by the stem cells using western blot analysis against rh-3αSHD. The immunoblot analysis was performed by washing (PBS/Tween20) and blocking (Licor), followed by incubation in primary (1:1,000 dil) and secondary (1/10,000). The membranes were scanned using Licor Odyssey system. As shown in **Fig. 6**, the transduced cells successfully produced the fused transgene (IL-2SS-3αHSD-GFP) (~64 kDa) as opposed to the rh-3αHSD alone.

Task-6: Investigate the *in vitro* ability of engineered ADMSC^{Sel} cells to metabolize androgens and suppress tumor cell growth, in CaP and/or in CaP/ADMSC^{AA} cocultures. 20-24

6.1. Monitor functional α-HSD expression by pLVX-IL-2SS-Akr1c14-IRES-GFP or the control LV constructs transduced ADMSCs by incubating ADMSC-CM with T or DHT and carry out androgen ELISA assays.

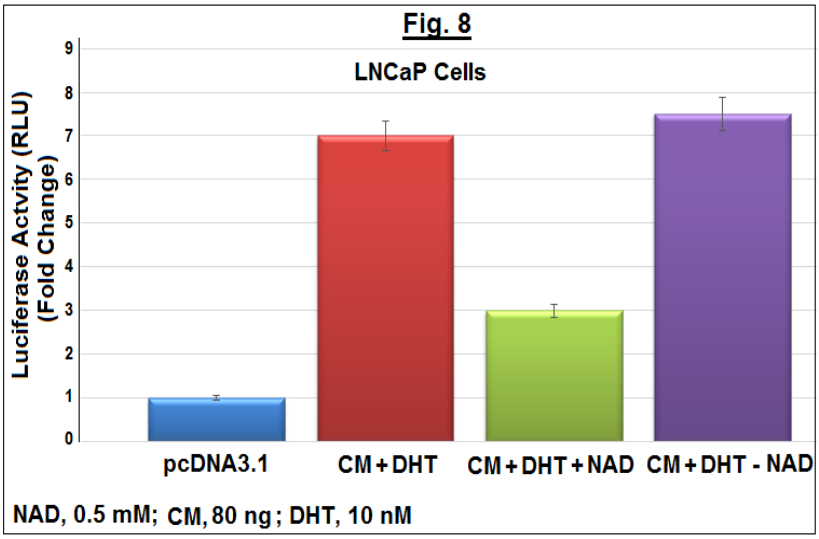
The functional significance of released 3αHSD by engineered ADMSCs in degrading/inhibiting DHT was explored using Cayman EIA Testosterone kit. Using 96-well plates, the conditioned medium (CM; 30 ng)

of the engineered stem cells or the rh3αHSD (20 ng) were added to the wells in triplicates in presence or DHT (0.1 nM) with or without NAD, required for 3αHSD activity.



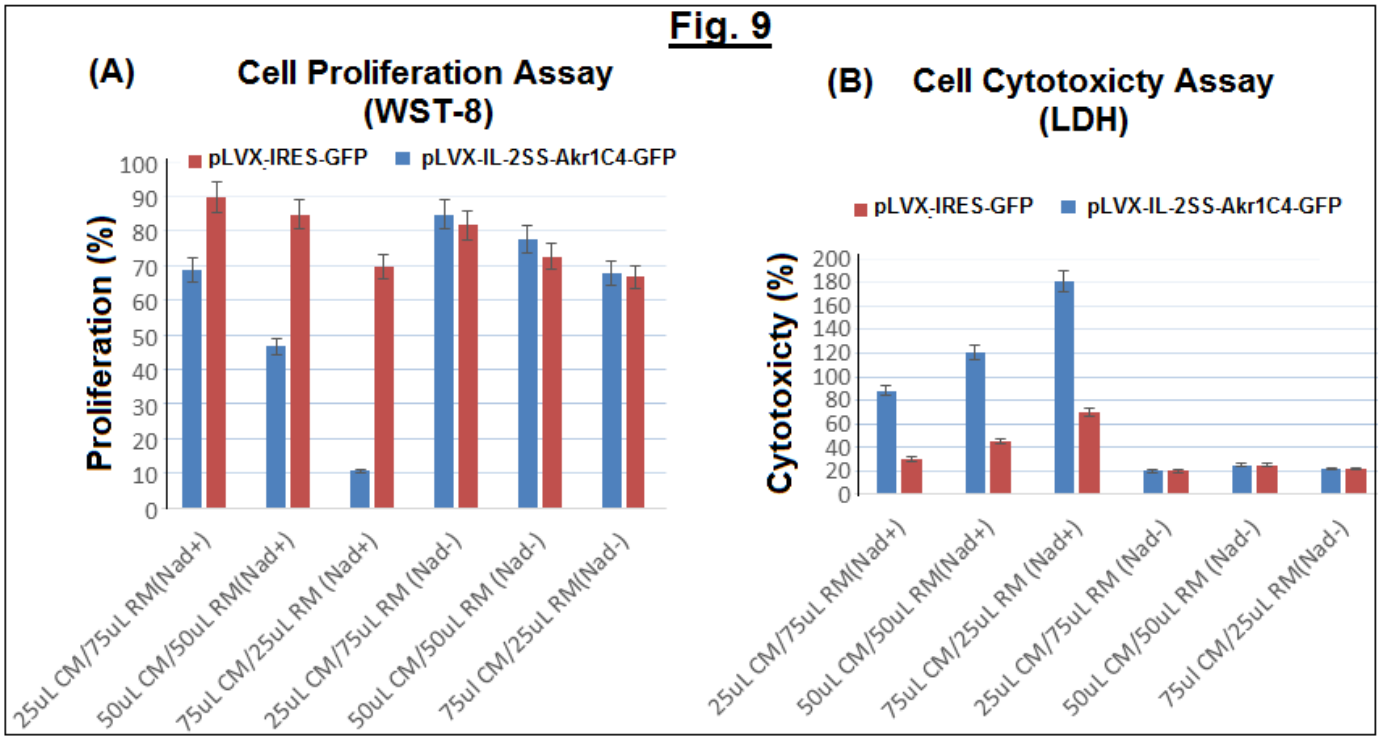
Shown in **Fig.7A** is the standard curve of testosterone or DHT ELISA. **Fig. 7B** depicts efficacy of the rh3αHSD in inhibiting DHT by approximately 50% at 20 ng concentration in presence, but not absence of NAD. Likewise, though to a lesser extent, the CM of engineered cells was able to suppress DHT by approximately 20% in presence of NAD at 30 ng concentration. The experiment, is preliminary, and would be repeated to optimize the amount of CM (a range of 20 to 100 ng) to achieve more potent inhibition of DHT by the transgene *in vitro*.

In a separate set of reporter assay experiments, we have examined the efficacy of CM of engineered stem cells in suppressing DHT induced AR *trans*activation in androgen dependent LNCaP cells. In these experiments we increased the concentration of CM to 80 ng based on weak response of 20 ng CM shown ion Fig. 7B. LNCaP cells were transiently transfected with a psPSA-luc plasmid, encompassing a PSA truncated promoter with AREs, or the control pcDNA3.1 plasmid, for 24 hrs. Cells were then treated conditioned media (CM) of engineered stem cells in presence or absence of DHT and NAD. As shown in **Fig. 8**, the DHT/AR activity was significantly suppressed (>50%) by CM in presence of NAD, suggesting it is effective at this or higher concentration to degrade/suppress DHT in the tumor microenvironment.



6.2. Coculture the GFP-labeled CaP cells with either control ADMSC^{Sel} or engineered ADMSC^{Sel} cells and compare growth rates in tumor cells, similar to Task-2.4.

6.3. Coculture the GFP-labeled CaP cells with ADMSC^{AA} cells similar to Task-2.4., and equal numbers of control ADMSC^{Sel} or engineered ADMSC^{Sel} cells and compare growth rates in tumor cells.

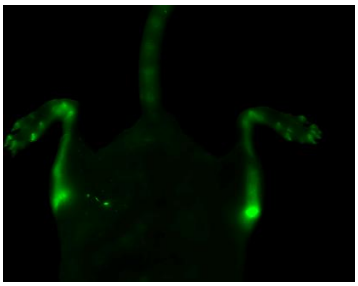


Next, we examined the ability of the conditioned medium (CM) of the enriched engineered stem cells (ADMSC^{Sel}) in inhibiting the growth and induce apoptosis of the androgen-dependent LNCaP cells *in vitro*. The cells were maintained to 50% confluency in 96-well plates in regular medium (RM) encompassing RPMI supplemented with 10%FBS. The medium was replaced with CM derived from ADMSCs transduced with control viral construct or Akr1C4 expressing construct containing various concentrations of CM (25 µl, 20 ng; 50 µl or 75 µl, 80 ng) in presence or absence of NAD. As shown in **Fig 9A**, the CM of Akr1C4-expressing cells showed a growth inhibition in a dose-dependent manner in presence of NAD, whereas no effect was observed in absence of NAD. The results suggest that 3αHSD was effective in inhibiting the growth of LNCaP cells through degradation of hormone in the medium. The results were corroborated by LDH cytotoxicity assays, demonstrating increased cell death by 3αHSD-containing medium in comparison to control media (**Fig. 9B**).

<u>SPECIFIC AIM-3</u>: Examine the efficacy of the therapeutically engineered ADMSC^{Cont} to target and inhibit CaP tumor cell growth in vivo (Months 20-36)

<u>Task-7</u>: Compare the ability of ADMSC^{Cont} and ADMSC^{Sel} cells to colocalize to bone tumor xenografts *in vivo*.

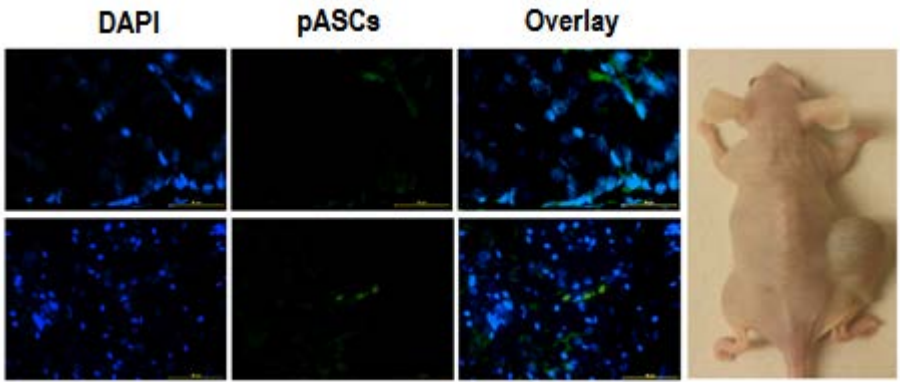
7.1. Inject CaP cells, alone or with ADMSC^{AA}, at intraosseous (femur) sites in nude-mice and monitor tumor development by tumor (~3-5 wks) by palpating and by serum PSA levels.



In Vivo Fluorescence Bone Imaging
(LiCOR Bone Dye Tag; 48 hr after Dye IP Injection)
Odyssey default Setting: Intensity 5.0, Focus 2.0 mm

Using standard technique, we transplanted the bone androgen independent PC-3 cells (positive control) or the androgen dependent LNCaP cells (2 x 10^6 cells) by direct intra-tibial injection in the femur of athymic nude mice. Four weeks later, a LiCOR Bone dye tag was injected i.v. and 48 hrs later, the mice were imaged by the LiCOR Odyssey machine. Shown in the above figure (previous page) is intraosseous tumor formation by PC-3 cells, but not LNCaP cells. In general, the androgen-independent LNCaP cells don't grow well in the in vivo setting, especially in bone. The above fining promoted us to transplant LNCaP s.c. to complete our proposed experiments.

7.2. Tail vein injection of labeled-ADMSCs (either ADMSC^{Cont} or ADMSC^{Sel} cells from same batch) and monitor whether the enriched ADMSCs cells preferentially engraft within the bone tumor foci. To optimize engraftment of the enriched adipose derived mesenchymal stem cells with osteotropism and tumor-homing potential (ADMSC^{Sel}), normal ADMSCs (ADMSC^{Cont}) were enriched by migration towards LNCaP prostate cancer cells conditioned media in a transwell system. The enriched ADMSC cells were transduced by a lentivitus construct expressing GFP (pLV-GFP). Nude mice (n=5) bearing LNCaP xenografts (8weeks) were injected with 2 x 10^5 transduced GFP-expressing ADMSCs and animals were sacrificed in 24 hours. Sown in the above figure are representative mouse bearing LNCaP xenofrat and tumor sections. The enriched ADMSCs (ADMSC^{Sel}) successfully engrafted in the LNCaP tumors, as indicated in the tumor sections by GFP and overlay immunofluorescence analysis.

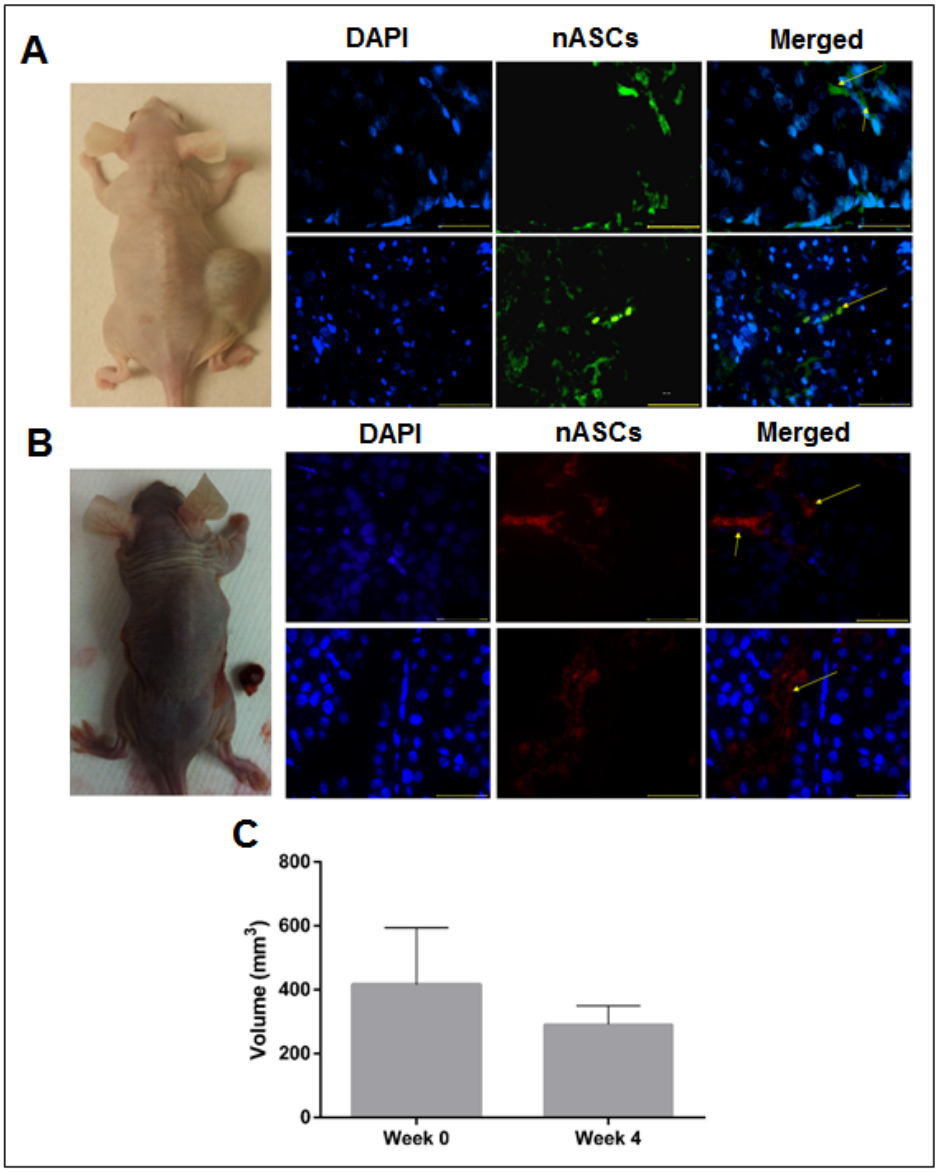


<u>Task-8:</u> Monitor the ability of LV-transduced ADMSC^{Sel} cells to suppress the CaP growth promoting effects of ADMSC^{AA} cells in tumor xenografts *in vivo*.

8.1. Investigate the effects of coinjected ADMSC^{Sel} cells producing α-HSD on tumor size in male nude-mice subcutaneously injected with CaP cells and ADMSC^{AA}

8.2. Determine the effects of engineered ADMSC^{Sel} cells injected via tail vein on growth of bone tumor xenografts containing CaP cells, alone or with ADMSC^{AA}

Because of inability of LNCaP cells to develop tumors in bone, we conducted these experiments in mice transplanted s.c. in athymice nude mice. We employed the strategy described previously (Applied Stem Cell, Inc.) to generate lentivirus expressing the pLVX-IL2SS- AKR1C14 -IRES-GFP gene product. A lentivirus expression vector, pLVX-IRES-ZsGreen1 (Clontech, Inc.) expresses two transgenes from a bicistronic mRNA transcript, allowing ZsGreen1 to be used as an indicator of transduction efficiency and a marker for selection by flow cytometry. The AKR1C14 (NM_138547) rat cDNA clone coding for 3α-hydroxysteroid dehydrogenase (Type I, 3α-HSD) was obtained from Origene. The IL2-SS (signal sequence) was synthesized (IDT, Inc.) to enable secretion of AKR1C14 enzyme by the recipient cells. Initially, the AKR1C14 gene was cloned in-frame with IL-2SS at the N-terminus in pCR-II plasmid using EcoRI and EcoRV restriction enzymes and adaptors and the DNA insert was sequence verified. The IL2SS-AKR1C14 sequence was PCR amplified with *Spe*I and *Not*I anchored primers. The PCR product was subcloned in SpeI-NotI digested pLVX-IRES-ZsGreen1 plasmid to generate pLVX-IL-2SS-Akr1c14-IRES-GFP construct. 5 x 10^4 normal ADMSCs (nASCs)/well were seeded in a 6 well plate and cultured overnight in regular stem cell media. Following morning, the wells were washed thrice with HBSS and the plate was put on ice. 400ul of Opti-Mem (Thermofisher Scientific, Rockford, IL) media containing 5ug/ml Polybrene from Millipore (Billerica, MA) was added to each well. The control GFP lentivirus and AKR1C14 lentivirus (2µl/well) was added to respective wells using pre chilled pipette tips. 24 hours later, the media was aspirated, wells washed with plain HBSS and regular stem cell media was added to the wells. The lentivirus uptake was visually established by examining GFP expression under Leica DMI3000B microscope (Leica Microsystems Inc., Buffalo Grove, Illinois) and immunoblotting in conditioned media as well qRT-PCR as

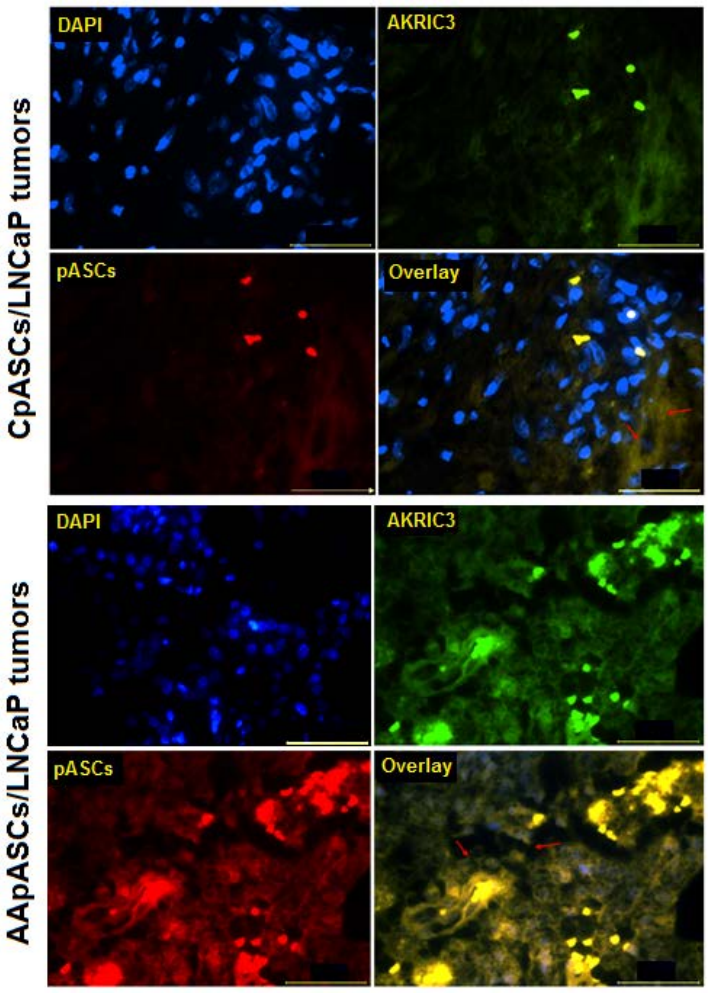


described before [26] (data not shown).

Next, we examined the tumor-homing potential of the enriched nASCs and transduced with AKR1C14-IL2SS-GFP lentivirus expression vector, and further examined their ability to regress tumor growth *in vivo*. Gonad-intact athymic nu/nu male (5-6 weeks old) (Taconic) were subcutaneously injected with LNCaP (4 x 10^6) cells suspended in 50 µL of serum-free RPMI-1640 medium and Matrigel (BD Bioscience, MD) (1:1). Upon tumor formation (~ 6 weeks), the animals were injected with the enriched pLV-AKR1C14-IL2SS-GFP transduced nASCs (1 x 10^6) via tail vein once every week for 4 weeks. As shown in the Figure A, the enriched nASCs effectively engrafted in the LNCaP tumors, as evidenced by GFP expression. Figure B depicts the expression of AKR1C14 protein (Texas Red color) in the LNCaP tumors. At the end of 4 weeks of treatment, the tumor volume was measured and recorded using the formula [0.5 x L x (W) ^{2}]. As shown in Figure C (previous page), a reduction of LNCaP tumors was observed in mice treated with AKR1C14-expressing nASCs compared to baseline tumor volumes. The results suggest that the engineered ADMSC can effectively target and inhibit growth of LNCaP tumor through secretion of androgen-degrading α-HSD in the tumor microenvironment.

8.3. Similar to Task-3.2., determine ADMSCs (mCherry) engraftment and local α-HSD expression in tumor bearing mice injected with either ADMSC^{Cont}, ADMSC^{Sel} or engineered ADMSC^{Sel} cells.
Please evidence of tumor engraftment of ADMSCs in Tasks 8.1 and 8.2 above.

8.4. Carry out similar experiments in castrated mice, to demonstrate that the tumor-homing of engineered ADMSC^{Sel} cells can target local androgens and suppress intraosseous tumor growth, even under ADT. We also demonstrated that the tumor-engrafted ADMSC^{AA} cells express androgen-metabolizing enzymes (AMEs), and presumably androgens, to support growth of the androgen-dependent LNCaP cells in castrated mice as shown I task 8.1 above. Shown on the figure (right) are representative immunofluorescence images of the AKR1C3 protein expression (red color) in formalin fixed paraffin embedded (FFPE) mouse tumor sections of LNCaP cells co-inoculated with GFP-expressing ADMSCs from CA men (*upper panels*) and LNCaP tumor -engrafted ADMSC^{AA} (*lower panels*) in castrated mice. The pASC engraftment was examined using anti-GFP antibody shown in red. The expression of AKR1C3 was detected using green-fluorescent Alexa Fluor® 488 secondary antibody. The nuclear stain is DAPI shown in blue (DAPI) and the co-localization of AKR1C3 and stem cells is shown by overlaying (*green arrows*) the images (*yellow color*). Scale bar= 50 µm.



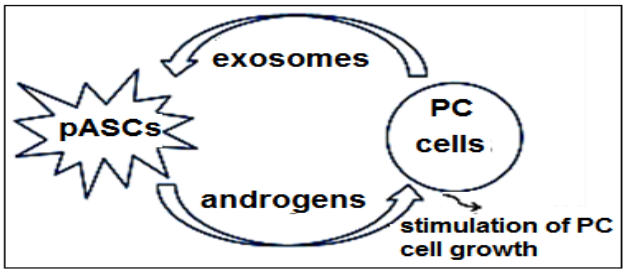
Once again, the results support the hypothesis that tumor-tropic ADMSCs from PC patients, especially AA-men, support growth of PC cells through *de novo* synthesis and production of androgens in the tumor microenvironment, even under androgen-deprivation conditions. This view was further strengthened by the expression of other key AMEs involved in de novo synthesis and production of androgens by ADMSCs co-inoculated with LNcaP cells (see Fig 11, first manuscript). Overall, the results suggest that tumor-tropic ADMSCs may support outgrowth of castration-resistant tumors through production of androgens in the tumor microenvironment.

What was accomplished under these goals?

We accomplished the milestones of the proposed research. The summary of the goals are:

(a) Mutual interactions between PC cells and tumor-recruited ADMSCs tumors are critical for upregulation of AME and intracrine androgen production in the tumor microenvironment.

(b) Further analysis of CM of PC cells, demonstrate that trafficking of PC cell-derived exosomes into the tumor-tropic ASCs are the culprits for upregulation of AME transcripts and de novo synthesis of androgen by these cells.



(c) The expression of AME/androgen production was significantly higher in ADMSCs derived from AA men compared to CA men. However, the underlying mechanisms remained to be elucidated.

(d) The tumor-tropic enriched ADMSCs pre-stimulated with PC cell-derived CM or exosomes augmented growth of LNCaP cells under hormone-deprivation conditions, both *in vitro* and *in vivo*. A robust effect was observed with ADMSCs derived from AA men than CA men. Our results support a new mechanism for tumor-tropic ADMSCs in supporting clonal expansion of metastatic prostate tumors among patients with CRPC.

(e) We constructed a bicistronic IRES lentiviral vector expressing the 3α-HSD by subcloning alpha keto reductase gene AKR1C14 (α-HSD) in frame with IL2-SS into the pLVX-IRES-ZsGreen1 vector (pLVX-3αHSD-IL2-SS-IRES-ZsGreen1). This enzyme is capable of targeting and degrading androgens.

(f) Tumor-homing normal ADMSCs transduced with the lentivirus construct effectively synthesized and secreted an active 3α-HSD enzyme, as measured by its ability to degrade androgens in vitro and inactivate its function in PC cells (reporter and survival assays).

(g) The enriched GFP-expressing AKR1C14 transduced nASCs successfully engrafted in tumors when administered in LNCaP tumor-bearing mice. A reduction in tumor volume, though not significant, was observed in mice administered with αHSD-expressing lentivirus construct compared to controls. Further optimization of the stem cell-based therapeutic approach, alone or in combination with hormonal therapy, may be required to achieved optimal response *in vivo*

(h) In conclusions, our data demonstrates that selective delivery of a αHSD by tumor-tropic ADMSCs can effectively hydrolyze residual androgens and potentially inhibit growth of PC cells under "castrate" conditions. To reduce residual androgens and achieve effective therapeutic response, further *in vivo* studies are required to optimize the dose and frequency of αHSD-expressing ADMSCs in tumor-bearing mice. The results suggest that αHSD-expressing nASCs can be incorporated as a mutli-modality hormone targeting therapy to reduce tumor burden in CRPC patients, especially among AA men.

The accomplished goals are summarized in the following two manuscripts:

First Manuscript

Patient-derived adipose stem cells primed with prostate cancer cell (PC)-associated exosomes confer prostate tumorigenesis through *de novo* synthesis of androgens under castrate conditions: Implications for PC progression among African Americans

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* M.R. and Y.Y. contributed equally to this work

Running Title: tumor-derived exosomes in *de novo* synthesis of residual androgens by stem cells

Keywords: Prostate cancer, stem cells, CRPC, steroidogenic enzymes, exosomes, intracrine androgen synthesis, tumor-tropic stem cells

Disclosure of Potential Conflict of Interest: The authors have no conflict of interest to disclose.

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Abstract

Background: The “intracrine” androgen production in the tumor metastatic microenvironment has been implicated in activation of functional androgen-signaling cascade and resurgence of castration-adapted prostate cancer (CRPC) under androgen deprivation therapy (ADT). Although mesenchymal stem cells have been shown to promote tumor growth, their role in producing residual androgens has not been investigated.

Methods: Patient adipose-derived mesenchymal stem cells (pASCs) were isolated from periprostatic fat was procured from PC patients undergoing radical prostatectomies. Conditioned media (CM) and their exosomes were collected from cultured androgen dependent, CRPC, and non-tumorigenic prostate cells using a standard protocol. The potential of PC derived CM and exosomes to induce transcriptional upregulation of key androgen-metabolizing enzymes (AMEs) and androgen production in pASCs and normal ASCs (nASCs) was verified by qRT-PCR and ELISA, respectively. Co-inoculation strategy was performed to examine the growth-inducing potential of fluorescently-labeled pASCs on androgen–dependent LNCaP cells in gonad-intact and castrated nude mice. The expression of AMEs in the tumor xenografts was monitored by immunofluorescence analysis and serum concentration was measured by ELISA.

Results: The CM of LNCaP cells stimulated transendothelial cell migration of pASCs *in vitro*. The LNCaP-derived CM triggered upregulation of AR, PSA and AME gene expression and testosterone production by pASCs, but not nASCs *in vitro*. Subsequent analysis demonstrated that PC cell-derived exosomes are responsible for AME gene expression and androgen production by pASCs. The enriched pASCs augmented growth of LNCaP cells under hormone-deprived conditions, both *in vitro* and *in vivo*.

Conclusions: Our results suggest that mutual interaction between PC cell-derived exosomes and tumor-tropic pASCs triggers AME expression and *de novo* synthesis of androgens in the tumor microenvironment. Our results support a new mechanism for tumor-tropic ASCs in supporting clonal expansion of metastatic prostate tumors among patients with CRPC.

Introduction

Prostate cancer in the most common and the second leading cause of cancer-related deaths among American males. According to SEER data (2009-2011), 15% men were predicted to be diagnosed prostate cancer (PC) in their lifetime. Many epidemiological studies have estimated a higher risk of prostate cancer in black men. African American men compared to the Caucasian Americans have a higher incidence of prostate cancer[1]. It has been argued that environmental, genetic factors and access to healthcare may be contributing factors [3]. Genetic mutations predisposing black men to prostate cancer have been identified by multiple studies while it has suggested that environmental factors such as diet and socio economic status influence the incidence rate [4-7]. However, very few studies have evaluated the relationship of the underlying tumor biology with the racial disparities of PC. Molecular data comparing the level of testosterone among the blacks and whites has generated conflicting results [8-10]. Furthermore, it has been shown that 5-α reductase may show a higher activity in black men compared to white men [11]. Therefore, further investigation into ethological and molecular factors in PC is required to determine the possible course for reducing disease burden.

Androgen-deprivation therapy (ADT) has been the mainstay therapy for patients with metastatic PC[12, 13]. Although initially effective, hormonal therapy is associated with an inevitable development of castration-resistant PC (CRPC) within 18 months and a median survival of 2–7 years. Emerging evidences are strongly indicative of transcriptional upregulation of endogenous steroidogenic enzymes and residual androgens responsible for eventual outgrowth of 'castration-adapted' tumors [14]. To this end, residual androgens were found to be 10–25% of circulating levels in untreated patients [15-17]. Indeed, despite 'castrate' serum levels, these prostatic residual androgens are sufficient [18] to activate androgen receptor (AR) signaling in tumors [17], thus implicating them in disease progression and development of CRPC. Post castration tissue dihydrotestosterone (DHT) decrease by 85% in patients with Gleason 6 tumors but only by 60% in patients with higher Gleason tumors[19], suggesting that modulation of tissue

androgen metabolism within the castrate tumors is critical for optimal and effective therapy. Although the source of residual androgens remains elusive, the source has been attributed to circulating adrenal androgens. Alternatively, it has been postulated that cholesterol may be a precursor for *de novo* biosynthesis of 'intracrine' androgens within prostatic tumors. The aforementioned observations attest that castration-adapted tumors may synthesize and maintain elevated testosterone for clonal expansion in anorchid patients with CRPC. Thus, in combination with conventional ADT, therapeutic strategies targeting endogenous androgens may have significant clinical implications for suppressing androgen signaling axis within the prostatic tumors to achieve optimal treatment response in patients with CRPC.

An epidemiological study established a link between adiposity (BMI > 25 kg/m^2) and risk of PC, disease progression and death [20]; however, the underlying mechanisms are not fully understood. As mesenchymal stem cells (MSCs), adipose-derived stem cells (ASCs) dwell within the perivascular niche of fat tissues and can be isolated from a heterogeneous cell population by differential adhesion of collagenase-digested stromal vascular fraction (SVF). ASCs have been shown to exhibit stable growth and proliferation kinetics and exhibit differentiation ability into multiple cell lineages [21]. Although such desirable traits make them attractive for clinical utility [22], the safe use of MSCs in regenerative and reparative medicine remains a matter of conjecture. This may be attributed to contradicting reports pertaining to the tumor promoting ability of normal MSCs[23]. Thus further research is required to unravel the *in vivo* underlying mechanisms by which MSCs promote tumor growth. The ability of adult MSCs derived from cancer patients to synthesize AME and androgens upon their recruitment and interaction with prostate tumor cells has not been explored.

Membrane vesicles (MVs) or exosomes are small intraluminal bodies derived from the endosomal compartment of cells under both normal and disease conditions [24]. Besides retaining their distinctive structural features, exosomes express an array of surface markers and their 'cargo' contents (miRNA, mRNA and proteins) are indicative of the cellular source from which they were originally derived [25]. A

number of exosomes endowed pleiotropic effects have been implicated in modulation of target cell biology and cell communications[26], primarily due to transmission of genetic materials to recipient cells[27, 28]. Although exosomes are implicated in tumor growth [25], their direct role in driving outgrowth of castration-adapted prostate tumors has not been elucidated.

In this study, we investigated the underlying molecular mechanisms by which tumor- tropic pASCs primed with PC cell-derived CM or exosomes synthesize intracrine androgens and promote PC growth *in vitro* and *in vivo* under androgen deprivation conditions.

MATERIALS & METHODS

Study population and fat tissue collection

In accordance with an approved IRB protocol and upon acquisition of informed consent, peri-prostatic fat tissue was collected from at least 15 patients with a mean preoperative PSA of 10.5 ng/ml, average age of 59.5 years, average Gleason score of 7.2 and average BMI of 32.4. Normal adipose tissue derived stem cells (nASCs) were a generous gift by Dr. Jeffery M. Gimble (Pennington Biomedical Research Center, Baton Rouge, LA).

Adipose stem cell isolation culture and preparation of conditioned media (CM)

Fresh periprostatic fat tissue (approximately 1 gm) procured from AA and CA men with PC was washed three times in PBS and minced on ice to approximately 1 mm^3 pieces as we previously described [22, 29]. The minced tissue was then suspended in 2 mg/ml of collagenase type-I (GIBCO, Invitrogen, Carlsbad, CA) constituted with PBS and subsequently incubated at 37°C in a shaking water bath for 2-2.5 hr. The cell suspension was then filtered through 70 µm and 40 µm cell strainer respectively (BD Biosciences, MD) to remove tissue debris. Mature adipocytes were removed by centrifugation (1,500x*g* for 10 min) followed by another wash in PBS. The resulting stromal vascular fraction (SVF) pellet is suspended and incubated for 2 min in red blood cell lysis solution (0.15 M ammonium chloride, 10mM potassium bicarbonate and 0.1 mM EDTA). Stem cells were washed in 2 ml 1% BSA (Sigma-Aldrich, MO), suspended in DMEM/F12 medium (1:1; v/v) supplemented with 10% FBS and 1% antibiotics- antimycotic solution (penicillin G, streptomycin and amphotericin B; Mediatech, Herndon, VA) and maintained at 37^oC with 5% CO_2. At 70% confluence, the nASCs and pASCs were cultured in phenol red free DMEM/F12, supplemented with 10% charcoal-stripped FBS

for 24 hr. Characterization of ASCs was performed as we described previously [29]. The CM was collected, filtered (0.2 µM) and stored at -20^{O}C until used.

PC cell culture and preparation of CM and exosome isolation

The nonmalignant, immortalized prostate cells (RWPE-1) and androgen-dependent LNCaP cells were purchased from ATCC (Manassas, VA). The isogenic CRPC C42-B cell line was a generous gift from Dr. L.W. Chung (Cedars-Sinai, Los Angeles, CA). LNCaP, C4-2B and PC-3 cells were cultured in RPMI-1640 medium (ATCC) supplemented with heat-inactivated 10% FBS and 1% penicillin/streptomycin (Invitrogen Life Technologies, MD). RWPE-1 was maintained in keratinocyte serum free media supplemented with EGF and bovine pituitary extract (Invitrogen Life Technologies, MD). For preparation of CM, both normal (RWPE-1) and PC cells were cultured up to 80% confluence in appropriate media following which they were washed thrice with DPBS and cultured in serum free, phenol red free and 1% penicillin/streptomycin for 24 hours. The CM was then collected and centrifuged at 1,500 RPM for 10 minutes, filtered (0.2 µm) and stored at -20°C until used. The exosomes from the CM were isolated as we described before [29].

Measurement of AME gene expression and androgen synthesis

The nASCs and pASCs were cultured in DMEM/F-12 supplemented with 10% charcoal stripped FBS up to 80% confluence. The ASCs were washed with PBS thrice and were cultured in Control serum-free medium, CM (1:1; v/v) or MVs (5-10 µg/mL) was prepared derived from RWPE1 or PC cells. The ASCs were then harvested at different time points (up to 96 hours) and the expression of AMEs (SRD5A1, SRD5A2, and AKR1C3 (as known as 17bHSD5), 17bHSD3, HSD17B1, HSD17B2, HSD17B3 and 3-β-HSD) in ASCs were analyzed by quantitative RT-PCR as described before [30]. Primer sequences were obtained from Getprime database [31] and ordered from IDT (Coralville, IA) (Table 1). A Testosterone EIA

was performed as per manufacturer's protocol (Cayman Chemicals, Ann Arbor, MI) to quantitate the release of testosterone. The results were normalized to controls and data was expressed as fold change ± SE from three independent experiments.

Animal studies

To examine the potential of pASCs to promote PC cell growth *in vivo*, the pASCs were transduced with eGFP and selected for puromycin (passage <5) and cryopreserved. Athymic *nu*/*nu* male mice (5-6 weeks old) (Taconics) were housed in a pathogen-free facility in accordance with approved IACUC animal protocol at Tulane University. To assess the effect of pASCs on PC cell growth *in vivo*, AApASCs or CpASCs (1×10^5) and LNCaP cells (4×10^6) were mixed in 100µL of serum-free DMEM medium and Matrigel (BD Bioscience, MD) (1:1 ratio) and then co-inoculated subcutaneously on flanks of gonad-intact and castrated mice. The mice were castrated via a midline scrotal incision as described [32]. The mice body weight and tumor sizes were recorded on weekly basis. The tumors were excised, weighed, and sectioned for immunofluorescence analysis.

Immunofluorescence analysis

Tumors were resected from euthanized mice as per approved protocol. Tumor sections were snap-frozen or paraffin-embedded for further analysis. Immunohistochemistry was performed as described before Tissue section were fixed on glass slide, de-paraffinization and antigen retrieval was performed at histology core facility in the Department of Pathology, Tulane University School of Medicine [33]. After the antigen retrieval the samples were blocked with 5% of goat serum in PBS and 0.2% Triton-X for 1 hour. Blocked samples were washed twice with 0.2% Triton X- 100 in PBS, then incubated (4°C, 12–16 h) with primary antibodies against GFP obtained from Genetex (Ann Arbor, MI). Tissue sections were then incubated for 1hr at room temperature in PBS 0.2% Triton X- 100 solution containing secondary antibodies conjugated with Alex Flour 488, 594, or 647- conjugated IgGs; (Molecular Probes, Invitrogen, Carlsbad, CA). Slides

were mounted with mounting medium with DAPI to stain the nuclei (Vectashield, Burlingame, CA). Images were acquired using Leica DMI3000B microscope (Leica Microsystems Inc., Buffalo Grove, Illinois).

Statistical Analysis

All experiments were conducted thrice and data generated was analyzed in SAS 9.4 software and Graphpad Prism 6.01. ELISA samples were quantitated by four way logistic regression and quantitated samples were compared using ANOVA. Post hoc analysis was performed using Sidak's multiple comparison and tukey test. Paired t-test was performed to compare the tumor volumes. Fold change in the qRT-PCR was estimated using Livak's fold change method and the estimated fold change was compared using non parametric ANOVA (Kruskal-Wallis test)

RESULTS

Androgen regulating genes are upregulated in AA-pASCs upon treatment with PC cell-derived CM

In order to examine the effect of exposure to tumor microenvironment on patient derived stem cells in context of race, a library of patient adipose derived stems cells (ASC) was established and racially categorized (African American patient ASC (AApASC) and Caucasian patient ASC (CpASC). 5 unique isolated AApASC & CpASC were randomly selected and then treated with CM from PC cells for 24 hours, following which mRNA from the cells were isolated and qRT-PCR was performed to examine the expression of androgen metabolizing enzymes. Up-regulation of mRNA expression in AApASCs and CpASCs compared to the normal stem cells (nASC) treated with same media, was observed in all AMEs examined encompassing AKR1C1, AKR1C2a, AKR1C2b, AKR1C3, SHD3B1, HSD17B1, HSD 17B3, SRD5A1, SRD5A2

(Figs 1-3). Interestingly, a significant ($P<0.05$) increase in transcript levels of AKR1C1, AKR1C3, SRD5A1, SRD5A2 were detected in AApASCs compared to CpASCs (Figs 1and 2).

Androgen-regulating genes are upregulated in pASCs upon treatment with exosomes derived from PC cells

It is known that exosomes are released by various cell types including cancer cells. These exosomes encapsulate different mRNA, miRNA, proteins etc. and are capable of transferring their content to other cells through several mechanisms. To narrow down the target components in PC cell CM which drive upregulation of AME in pASCs, we isolated exosomes from CM of PC cells (LNCaP, C4-2B) and RWPE-1 CM as described in methods and treated AApASC & CpASC lines with physiological concentration of MVs for 24 hours [29], mRNA from these cells were isolated and qRT-PCR was performed to examine the expression of AMEs. Up-regulation of mRNA expression in AApASCs and CpASCs compared to the nASC control was observed in all the AMEs examined (Figs 3-6). A higher fold change upregulation of AME expression was observed when stem cells were treated with cancer cells derived

exosomes compared to non-tumorigenic RWPE-1 cells derived exosomes (Figs 3-6). Similar differences in the transcript levels of AKR1C1, AKR1C3, SRD5A1, SRD5A2 were detected in AApASCs compared to CpASCs (Figs 3 and 4).

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AA-pASCs show higher testosterone secretion upon treatment with PC cell-derived CM or exosomes

To verify the effect of AME upregulation on testosterone concentration *ex vivo*, AApASCs and CpASCs were treated with CM derived from PC cells or non-tumorigenic cells for 24 hours and DHT concentration was estimated using testosterone ELISA kit. Exposure of pASCs to LNCaP CM lead to a higher secretion of testosterone compared to the exposure to CM derived from non- tumorigenic cells and C4-2B cells (Figure 7A). Importantly, AApASCs showed a significantly higher average concentration of testosterone compared to CpASCs upon exposure to PC cell-derived CM ($p<0.05$) (Figure 7A). In order to investigate the *ex vivo* effect of PC cell-associated exosome treatment on the *de novo* synthesis and release by AApASC and CpASC, the cells were treated with physiologic concentrations of exosomes derived from PC cells or non-tumorigenic cells for 24 hours and testosterone concentration was estimated using ELISA kit. As shown in Figure 7b, testosterone concentration is significantly high ($P<0.05$) in pASCs treated with exosomes derived from LNCaP cells compared with cells treated with exosome derived with from non-tumorigenic cells and C4-2B cells. It was further determined that AApASC show a higher concentration of testosterone compared to CpASC after exposure MVs ($p<0.05$) (Fig. 7b).

AApASCs co-inoculated with androgen-dependent LNCaP cells form larger tumors in gonad-intact and castrated mice

Next, we examined if residual hormones released by pASCs procured from AA and CA men (n=5 different isolates per group) differentially modulate growth of androgen-dependent LNCaP cells *in vivo*. To mimic the castration adapted state, gonad-intact and castrated mice were co-inoculated with LNCaP cells and either AApASCs or CpASCs on the left and right flanks of the same mouse (n=5), respectively. Tumors were allowed to form and were then resected. Figures 8A and B show representative images of LNCaP tumors formed in gonad-intact and castrated mice, respectively. Regardless of the blood circulating

status, LNCaP formed larger tumors when co-transplanted with AApASCs compared to compared to CpASCs (Fig 8 A, B). As measured by an EIA, the circulating levels of testosterone were significantly lower in castrated mice compared with gonad intact mice (Fig. 8C). Significantly larger LNCaP tumor weights were detected in mice co-transplanted with AApASCs compared to those co-inoculated with CpASCs in both gonad-intact (Fig. 8D) and castrated (Fig 8E) mice.

AA-pASCs co-inoculated with androgen dependent LNCaP cells tumor sections show higher expression of AMEs compared to CA-pASCs in gonad intact and castrated mice.

In order to examine the hypothesis that PC cells trigger higher AMEs expression in AApASCs *in vivo*, IHC was performed on the resected tumor sections. Formalin fixed paraffin embedded tumor sections were taken from both gonad intact and castrated mice bearing AApASC/LNCaP tumors or LNCaP/CpASC tumors on either flank. As shown in Figure 9, the protein expression of AMEs, exemplified by AKR1C3, in AApASC/LNCaP tumors was higher compared with CpASC/LNCaP tumors in gonad-intact mice. Likewise, the protein expression of AKR1C3, was found to be higher in AApASC/LNCaP tumors compared with CpASC/LNCaP tumors in castrated mice (Fig. 10). Further analysis also noted abundant engraftment of GFP-expressing AApASCs in LNCaP tumors compared to CpASCs (Figs 9 and 10). Analysis of mean fluorescence intensity as quantitated by ImageJ (ver 1.49) was used as a function of AMEs protein expression. The results demonstrated significantly higher expression ($p<0.05$) of AKR1C1, AKR1C3, SRD5A1 and SRD5A2 in AApASC/LNCaP tumors than in CpASCs/LNCaP tumors both in gonad-intact (Fig 11A) and castrated mice (Fig 11B).

Discussion

It has been reported before that the AMEs are upregulated in the tumor tissue compared to the normal adjacent tissue (NAT) [34]. The expression of AMEs can be regulated by the tumor micro environment. AA men when diagnosed with prostate cancer often show a worse prognosis compared to their Caucasian counter parts. A difference in tumor micro environment may be a contributing factor leading to worse prognostic outcome.

In this study, we demonstrated for the first time, the mutual interaction between patients' derived adipose stem cells and PC cells may potentially enhance tumor burden and expansion in patients under androgen ablation therapy. The growth promotion effect is initiated by exosomes released by PC cells upon recruitment of pASCs to the tumor microenvironment. Isolated exosomes from PC cells, but not RWPE-1cell derived exosomes, triggered gene expression of AMEs and de novo synthesis of androgens by pASCs in a manner similar to that primed by PC cell CM., but with abundant response from AApASCs than CpASCs. This difference was also reflected in higher testosterone release by AApASCs compared to CpASC upon exposure to exosomes derived from androgen dependent LNCaP cells under androgen-deprivation conditions. These results support the notion that tumor-recruited pASCs may enable PC cells to enable growth advantage and castration adapted state in patients receiving androgen ablation therapy. Exosomes are considered major endogenous "signalomes' in terms of mediating cell-cell communications and in modulating biology of the recipient cells through trafficking of transcripts, gene-regulatory factors and proteins into recipient cells. Considering the variety of exosome "cargo' contents of nucleic acids and proteins, it remains to be elucidated if exosome trafficking of mRNAs, miRNA or proteins, alone or in concert, play a role in expression of AMEs in the recipient pASCs. We previously demonstrated that part of the tumor-recruited pASCs, but not nASCs, may contribute to tumor clonal expansion through their tumor mimicry and neoplastic reprogramming [29]. The present findings suggest that tumor-homing

pASCs may possess diverse and unique intrinsic properties and influence tumor growth and expansion through various mechanisms, including de novo hormone synthesis and oncogenic reprogramming.

Disproportionate prostate tumor volume and disease progression has been reported in AA compared to CA men and other ethnic minority groups in the U.S. The present study, for the first time, highlights differences in influencing AMEs gene expression and *de novo* synthesis of androgens in pASCs, but not nASCs. Importantly, we also demonstrated abundant AMEs gene expression and *de novo* synthesis in AA patients' derived ASCs compared to CA men-derived counterparts when triggered by PC cell derived exosomes. The androgen production by AApASCs significantly enhanced growth of the androgen-dependent LNCaP tumors in gonad-intact and castrated nude mice. Based on our immunofluorescence analysis, the racial differences in the tumor growth promoting ability may be attributed to substantial and intrinsic ability of AApASCs , as opposed to CpASCs, to engraft in LNCaP tumors *in vivo*. These results suggest that androgen ablation therapy may trigger recruitment of tissue resident and/or circulating ASCs to facilitate *de novo* androgen synthesis and allow survival of androgen-dependent prostate tumors under a castration-adapted state.

Taken together, the present study provides a new role for tumor-homing ASCs in expression of AMS and *de novo* synthesis of androgen upon interaction with androgen-dependent PC cells *in vitro* and *in vivo.* As residual androgens, the results also highlight the potential role they may play in supporting growth of outgrowth of "castrate-adapted" prostate tumors in CRPC patients. These results may also provide an explanation for disease progression and worse prognosis in AA men. In combination with conventional androgen deprivation therapy, therapeutic strategies aimed at eliminating residual androgens may have significant clinical implications to circumvent androgen signaling axis within the prostatic tumors to achieve optimal treatment response in patients with CRPC, especially among AA men.

Acknowledgements

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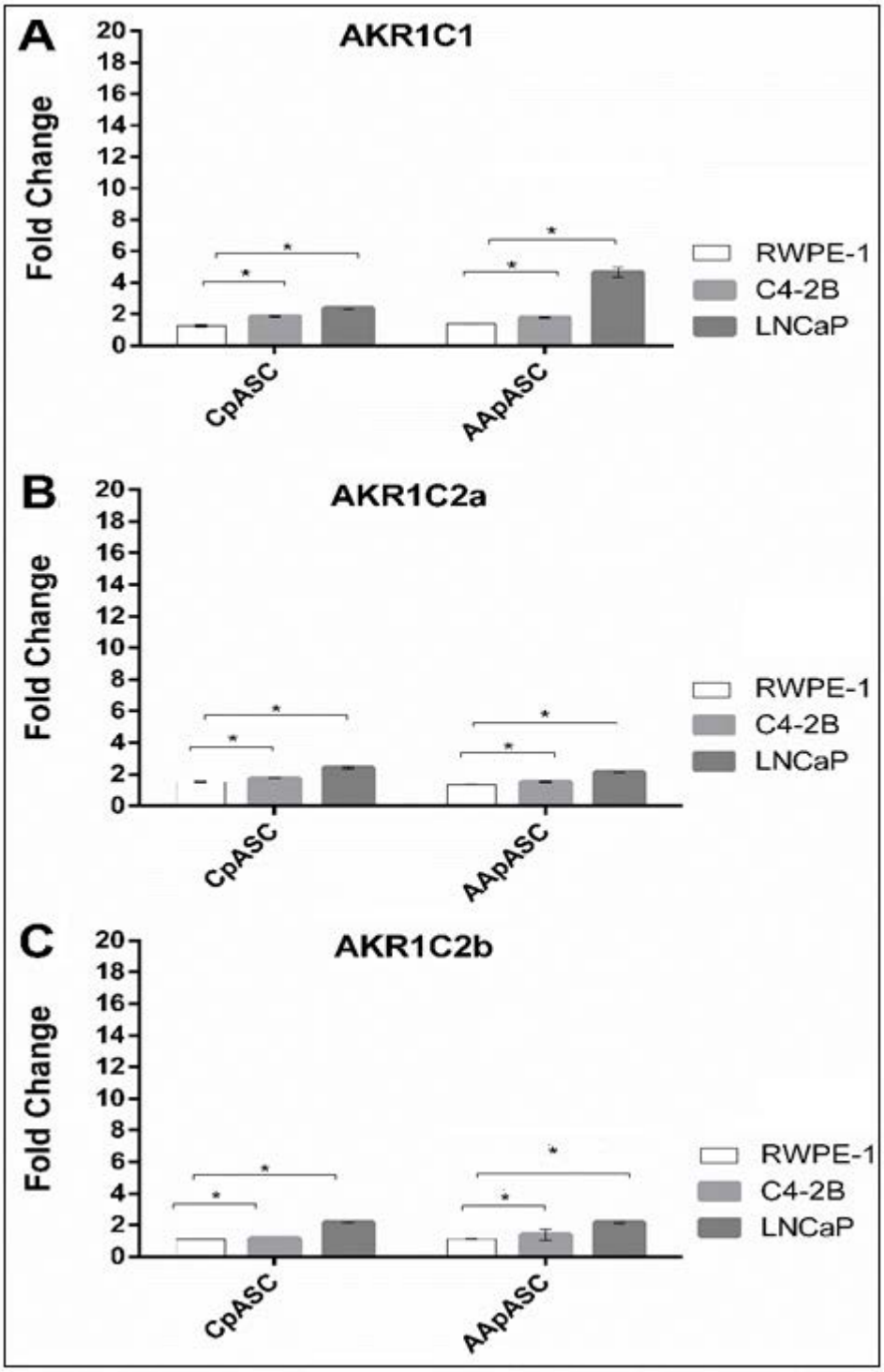

Figure 1. Fold change upregulation of androgen-metabolizing enzyme (AME) genes (A, AKR1C1; B, AKR1c2a; C, AKR1C2b) in AA-pASCs and CpASCs upon treatment with PC cell-derived CM. Fold change was calculated using Livak's method. Calculated fold change was compared using non parametric ANOVA (Kruskal Wallis test) followed by post hoc analysis using a Tukey test and significance was determined at $p<0.05$.

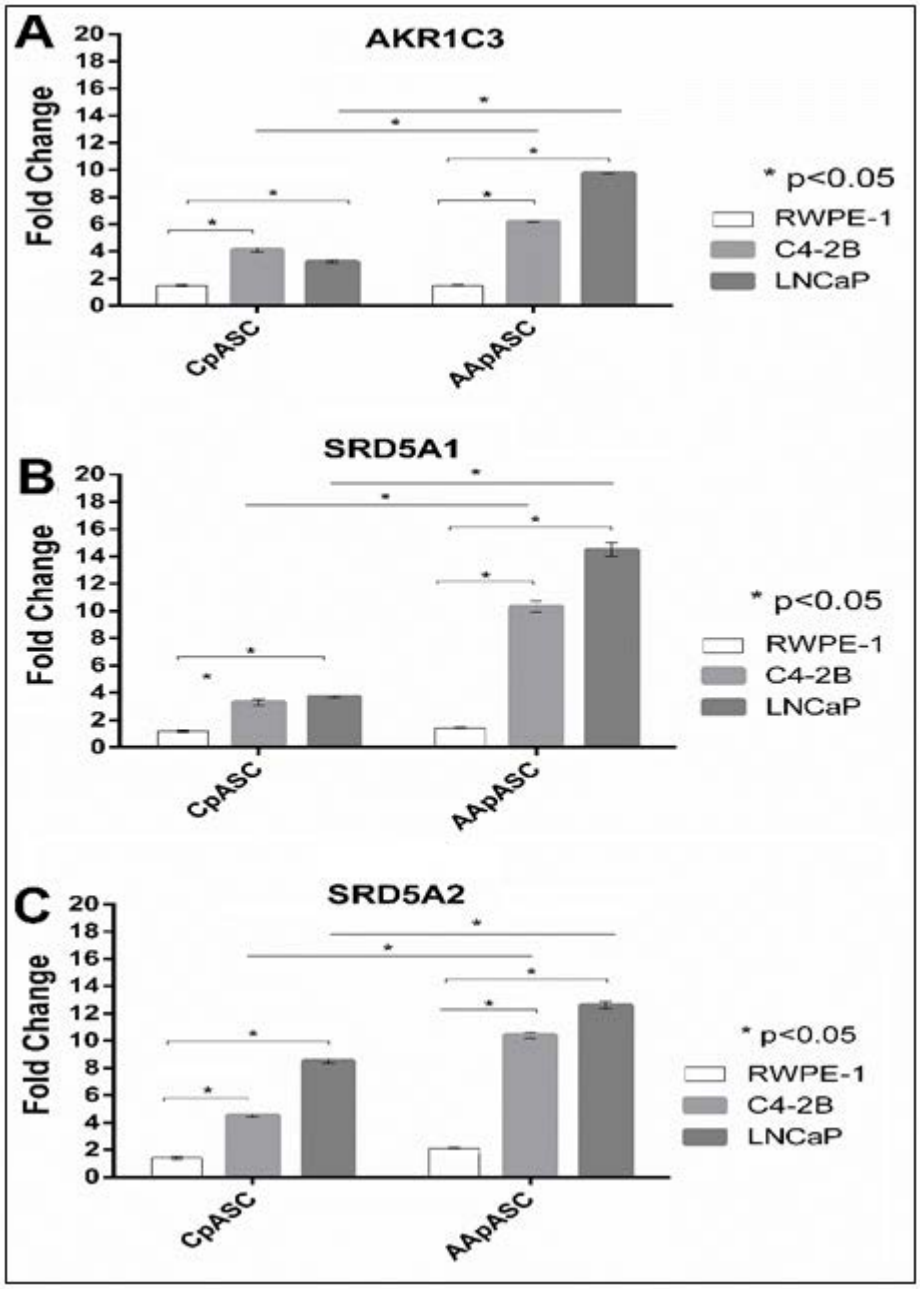

Figure 2. Fold change upregulation of androgen-metabolizing enzyme (AME) genes (AKR1C3, SRD5A1 and SRD5A2) in AApASCs and CpASCs upon treatment with PC cell-derived CM. Fold change was calculated using Livak's method. Calculated fold change was compared using non parametric ANOVA (Kruskal Wallis test) followed by post hoc analysis using a Tukey test and significance was determined at $p<0.05$.

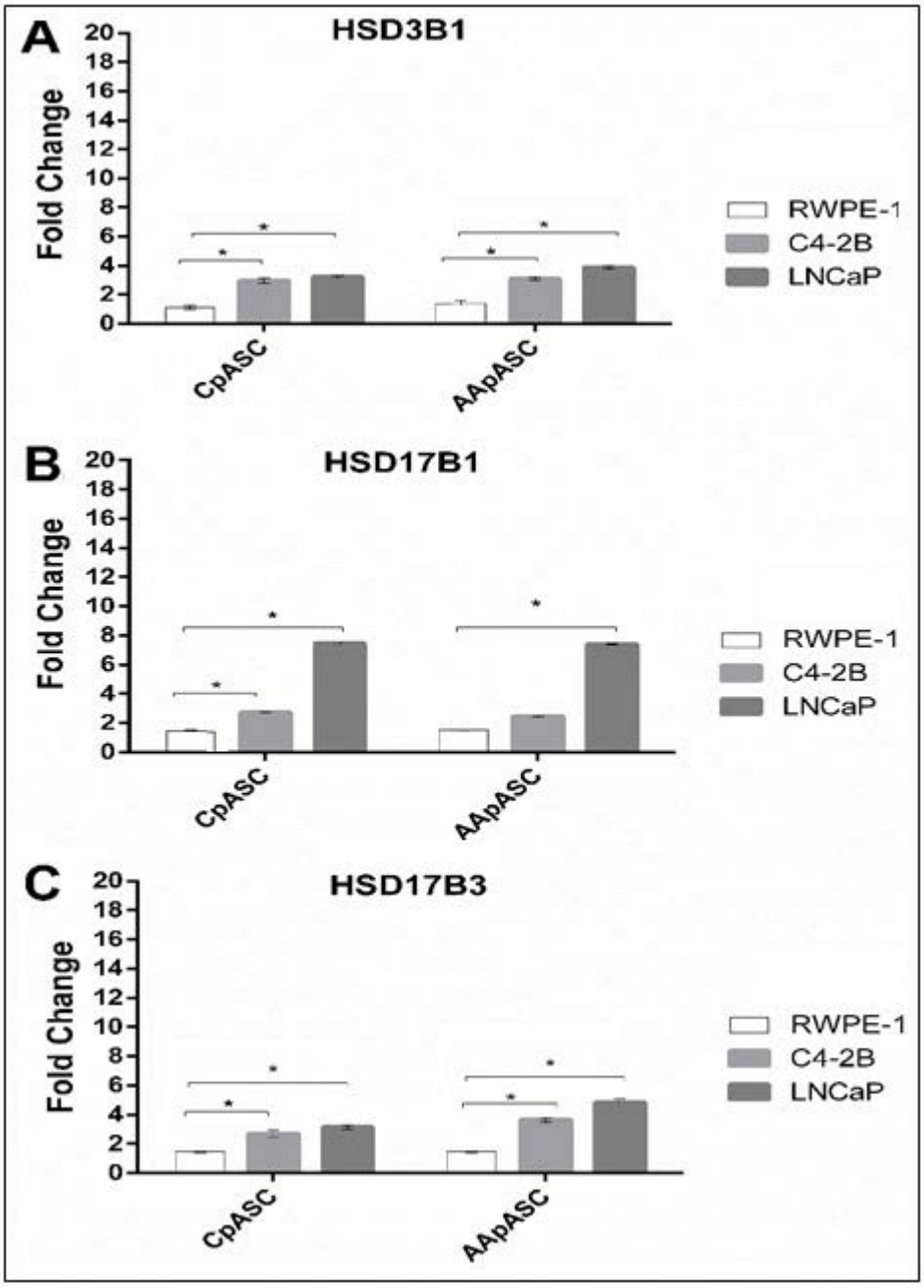

Figure 3. Fold change upregulation of androgen-metabolizing enzyme (AME) genes (A, HSD 3ß1; B, HSD 17ß1; C, HSD17ß3) in AA-pASCs and CpASCs upon treatment with PC cell-derived CM. Fold change was calculated using Livak's method. Calculated fold change was compared using non parametric ANOVA (Kruskal Wallis test) followed by post hoc analysis using a Tukey test and significance was determined at $p<0.05$.

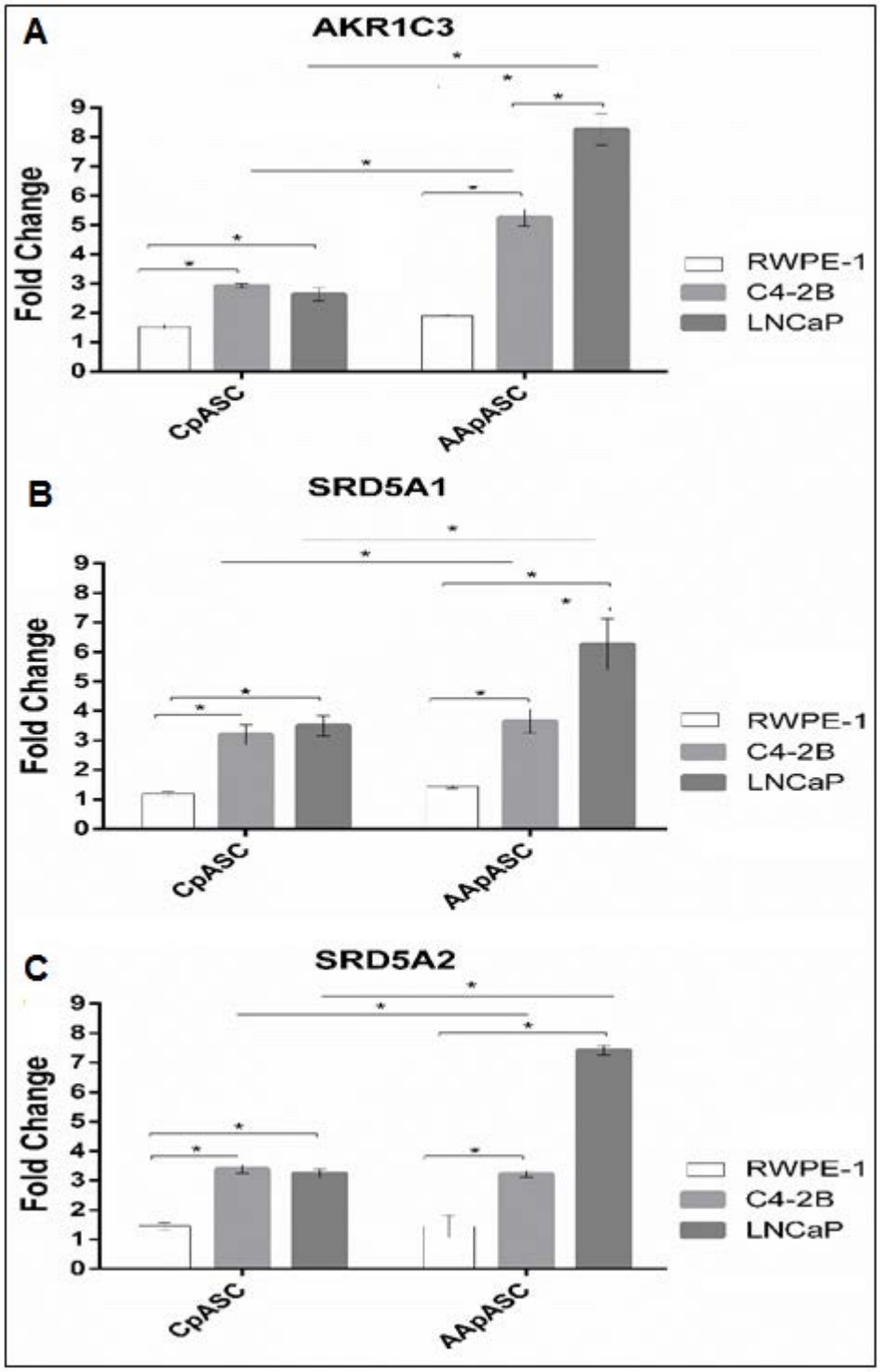

Figure 4. Fold change upregulation of androgen-metabolizing enzyme (AME) genes (A, AKR1C1; B, AKR1c2a; C, AKR1C2b) in AA-pASCs and CpASCs upon treatment with PC cell derived exosomes. Fold change was calculated using Livak's method. Calculated fold change was compared using non parametric ANOVA (KruskalWallis test) followed by post hoc analysis using a Tukey test and significance was determined at $p<0.05$.

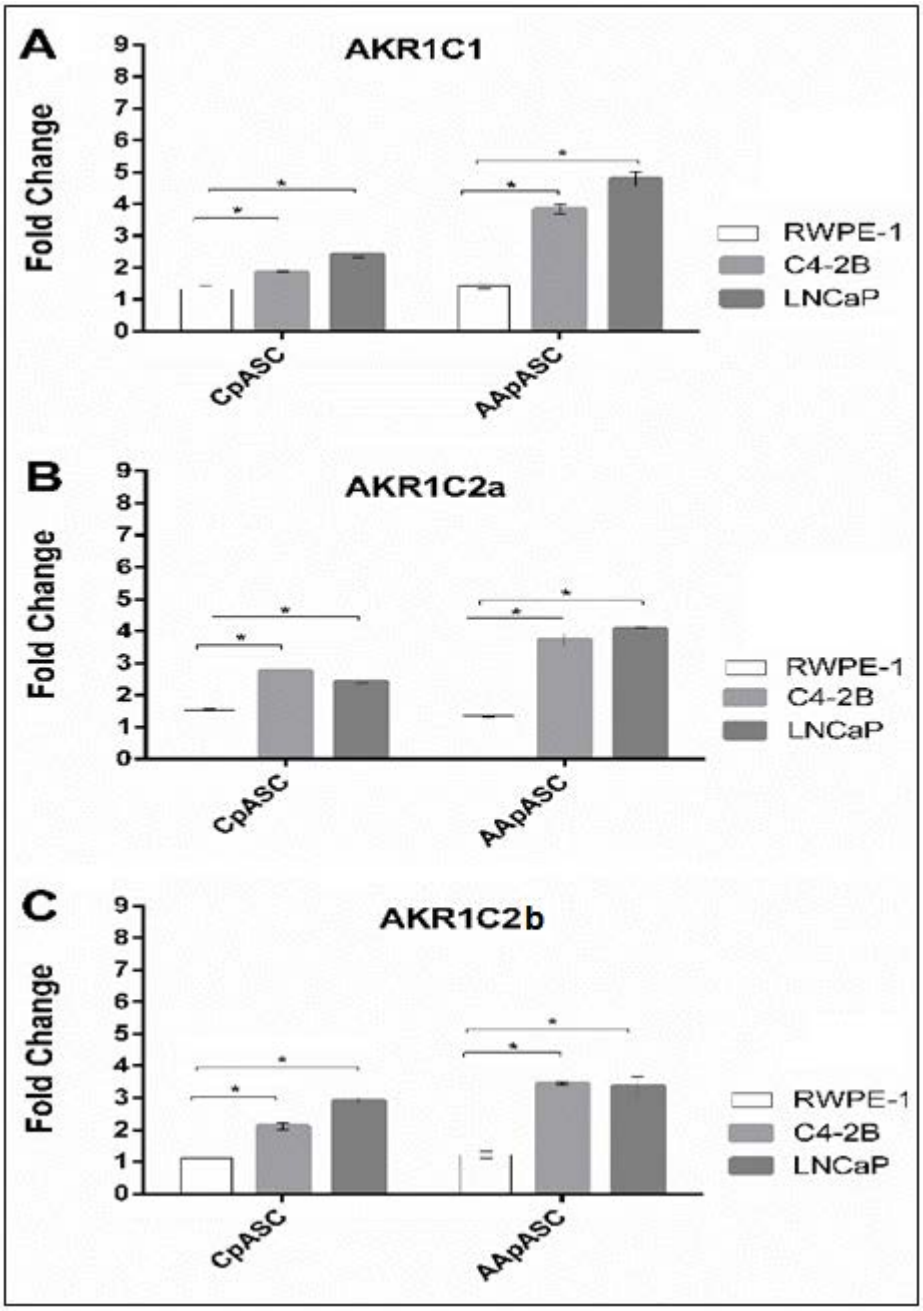

Figure 5. Fold change upregulation of androgen-metabolizing enzyme (AME) genes (A, AKR1C3; B, SRD5A1; C, SRD5A2) in AA-pASCs and CpASCs upon treatment with PC cell derived exosomes. Fold change was calculated using Livak's method. Calculated fold change was compared using non parametric ANOVA (KruskalWallis test) followed by post hoc analysis using a Tukey test and significance was determined at $p<0.05$.

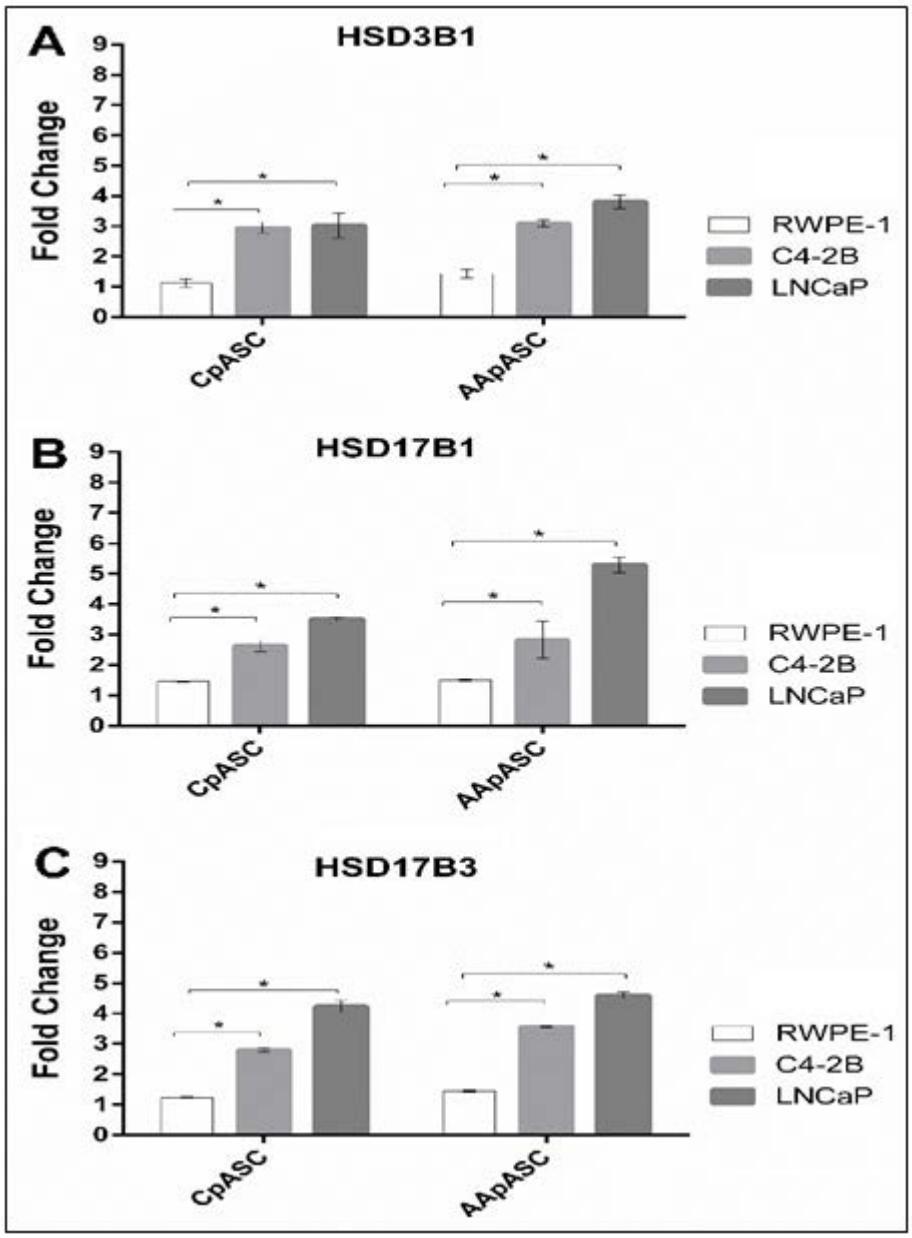

Figure 6. Fold change upregulation of androgen-metabolizing enzyme (AME) genes (A, HSD 3ß1; B, HSD 17ß1; C, HSD17ß3) in AA-pASCs and CpASCs upon treatment with PC cell- derived exosomes. Fold change was calculated using Livak's method. Calculated fold change was compared using non parametric ANOVA (KruskalWallis test) followed by post hoc analysis using a Tukey test and significance was determined at $p<0.05$.

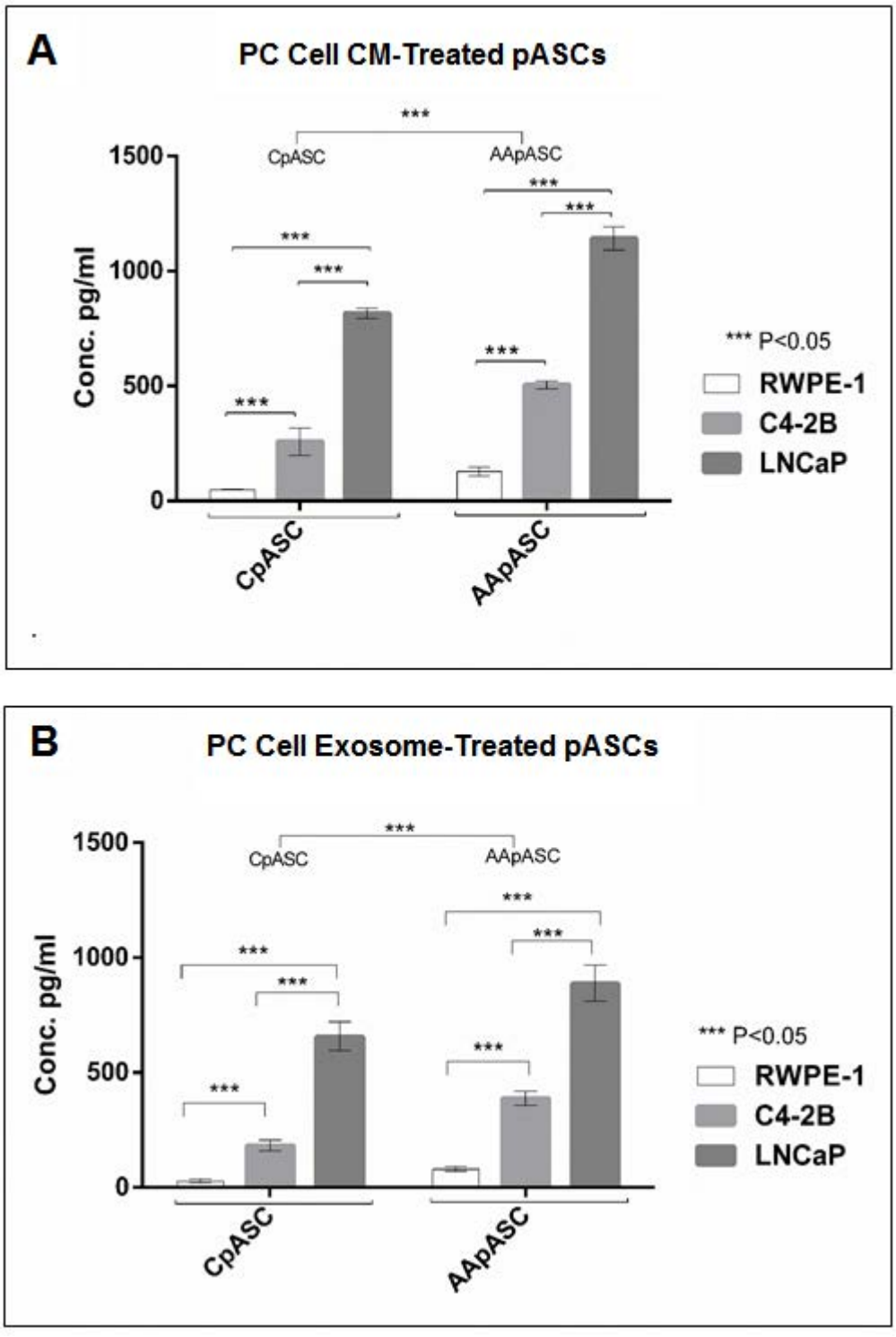

Figure 7. Testosterone secretion quantitated by EIA shows higher concentration in AApASCs compared to CpASCs when cells treated with PC-derived CM or exosomes. Data was normalized to nASCs and pASCs treated with RWPE1 cell-derived CM or exosomes. *** denote significance at $p<0.05$.

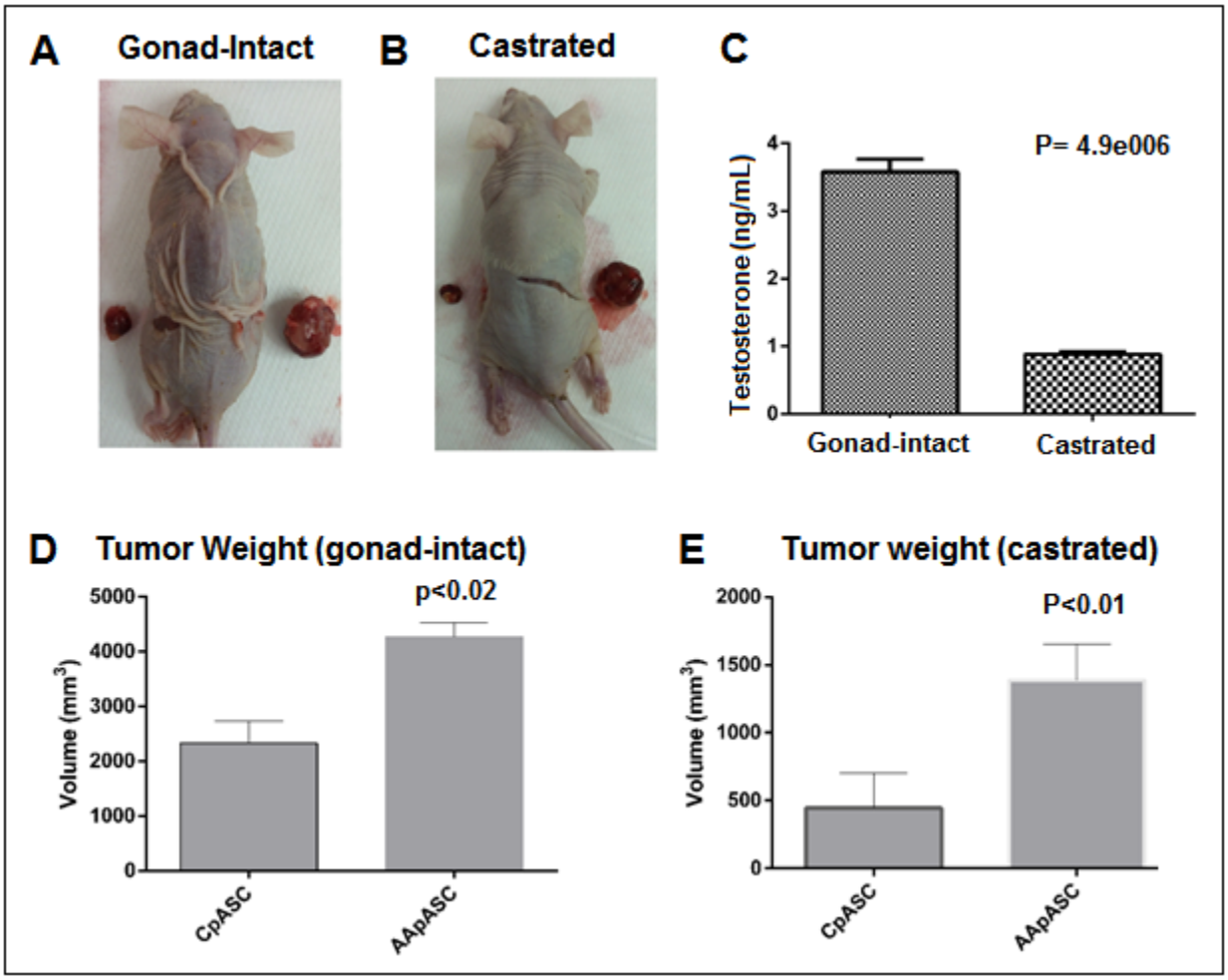

Figure 8. AApASCs support LNCaP tumor growth in gonad-intact and castrated nude mice. Representative images of tumor volumes of LNCaP tumor formation co-transplanted with GFP-expressing AApASCs (right flank) or CpASCs (left flank) in gonad intact (A) and castrated (B) mice. C, depicts serum concentration of testosterone as estimated by ELISA was significantly lower in castrated mice compared to gonad-intact mice (p value<0.001). LNCaP cells co-inoculated with AApASCs showed significantly higher tumor volumes than the LNCaP cells co-inoculated with CpASCs in both in gonad-intact and castrated mice. LNCaP cells co-inoculated with AApASCs showed significantly higher tumor weight than the LNCaP cells co-inoculated with CpASCs in both in gonad-intact (D) and castrated mice (E).

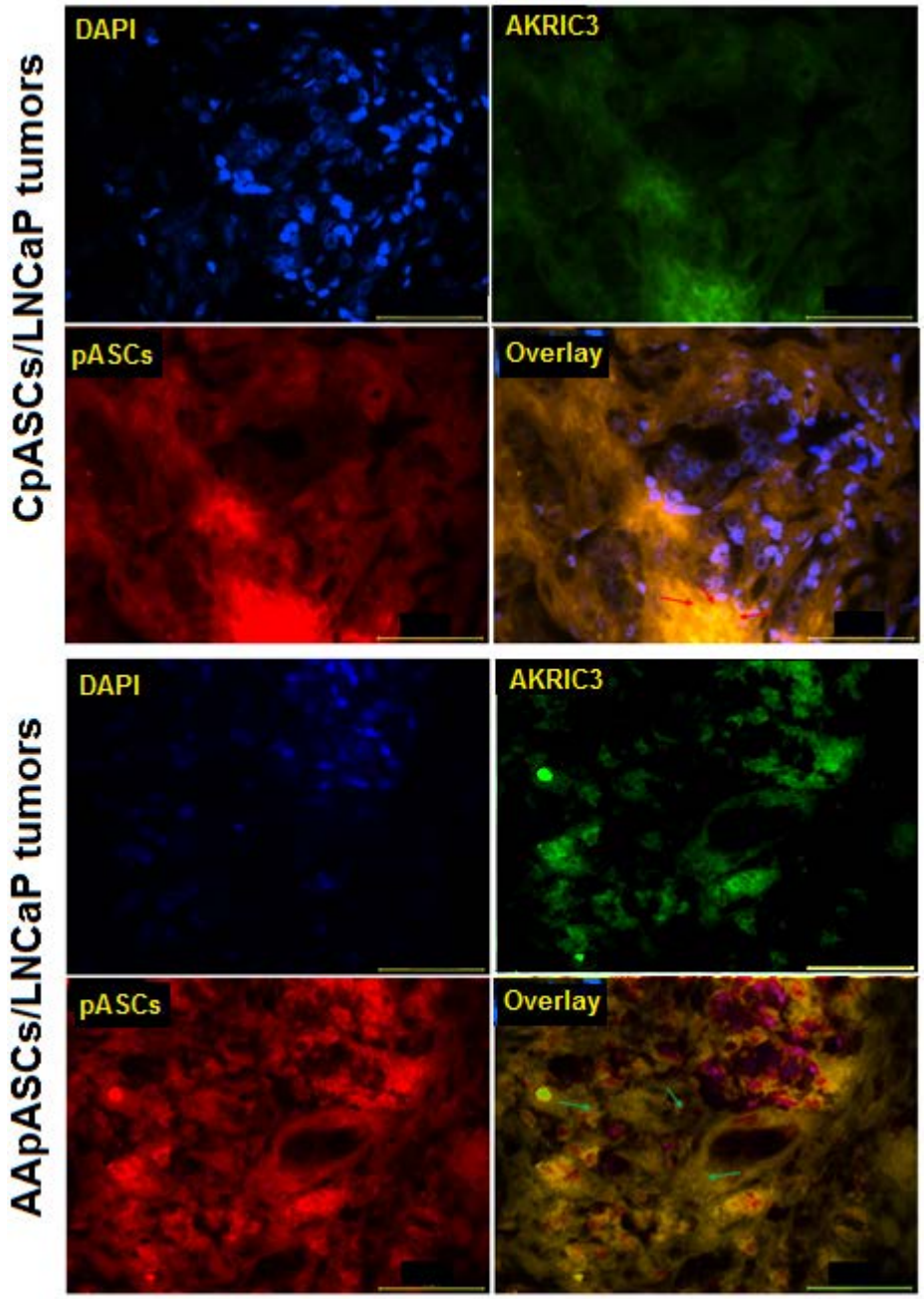

Figure 9. Representative immunofluorescence images of AKR1C3 protein expression (red color) in formalin fixed paraffin embedded (FFPE) mouse tumor sections of LNCaP cells co-inoculated with GFP-expressing CpASCs (*upper panels*) and LNCaP tumor-engrafted AApASCs (*lower panels*) in gonad-intact mice. The pASC engraftment was examined using anti-GFP anti-body shown in red. The expression of AKR1C3 was detected using **green**-fluorescent **Alexa Fluor**® 488 secondary antibody. The nuclear stain is DAPI shown in blue (DAPI) and the co-localization of AKR1C3 and stem cells is shown by overlaying (*green arrows*) the images (*yellow color*). Scale bar= 50 µm.

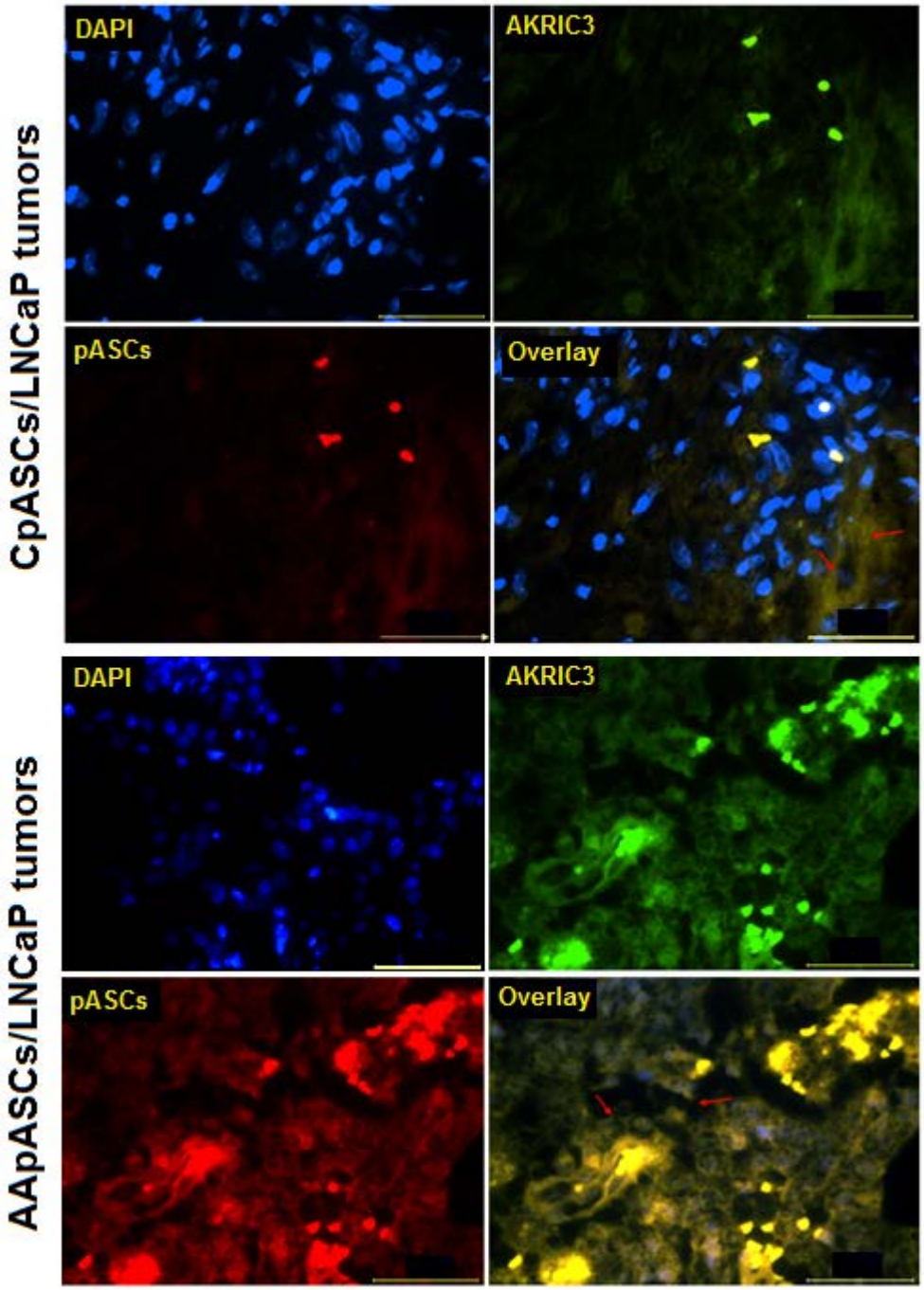

Figure 10. Representative immunofluorescence images of AKR1C3 protein expression (red color) in formalin fixed paraffin embedded (FFPE) mouse tumor sections of LNCaP cells co-inoculated with GFP-expressing CpASCs (*upper panels*) and LNCaP tumor -engrafted AApASCs (*lower panels*) in castrated mice. The pASC engraftment was examined using anti-GFP anti-body shown in red. The expression of AKR1C3 was detected using **green**-fluorescent **Alexa Fluor**® 488 secondary antibody. The nuclear stain is DAPI shown in blue (DAPI) and the co-localization of AKR1C3 and stem cells is shown by overlaying (*green arrows*) the images (*yellow color*). Scale bar= 50 µm.

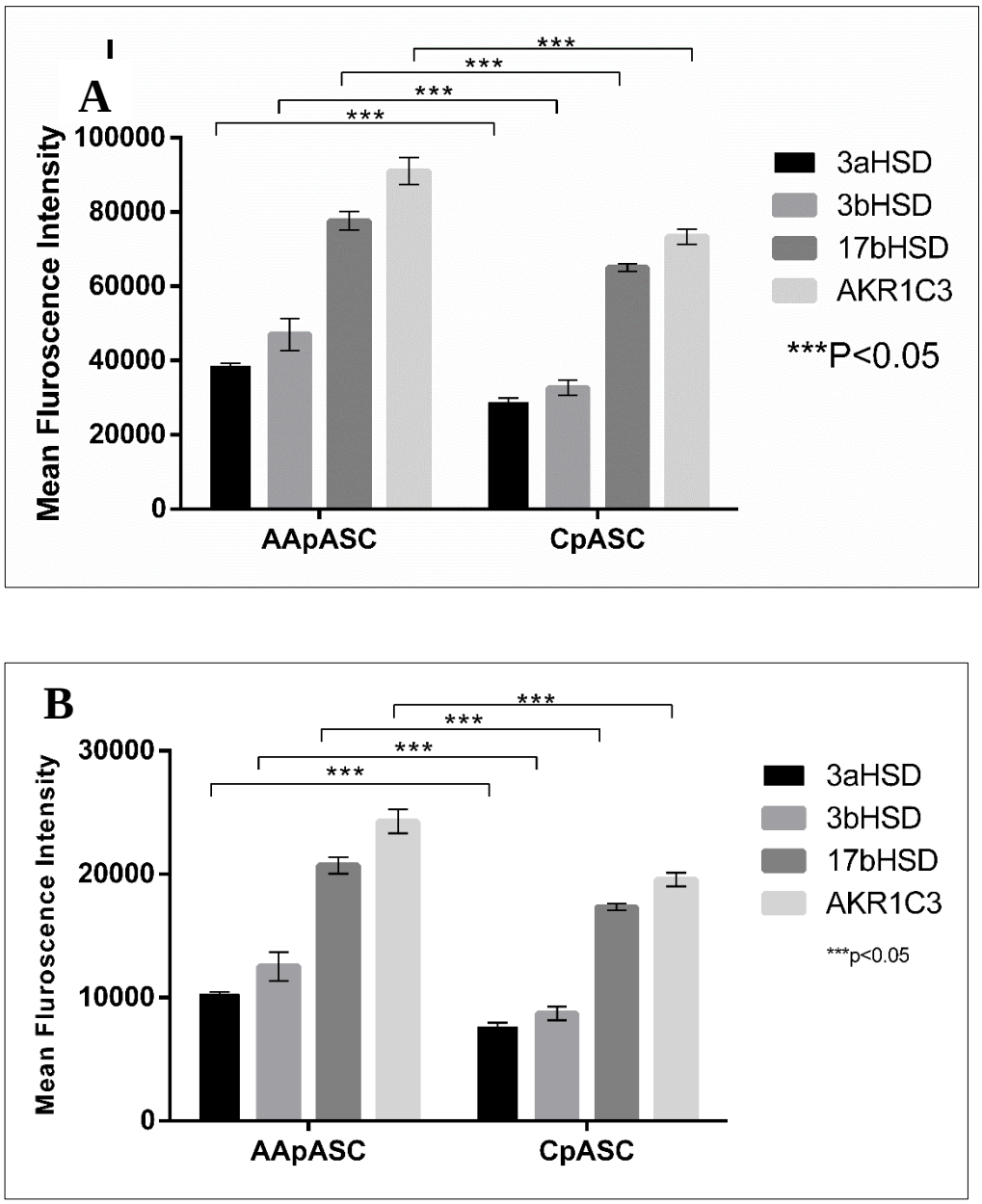

Figure 11. IHC quantitative analysis (ImageJ) of AMEs protein expression in the engrafted pASCs in FFPE mouse LNCaP tumor sections in gonad intact (A**)** and castrated (B) mice. The mean fluorescence intensity was used as a function of expression of AMEs in the IHC sections and was calculated using ImageJ software. *** denotes significance at P<0.05.

Table 1: Primer sets for qRT-PCR

Gene	Forward sequence	Reverse sequence
AKR1C1	AATTGGCAATTGAAGCTGG	CTCACCTGGCTTTACAGAC
AKR1C2a	ATGATCCTCAACAAGCCAG	AAGGATGACATTCCACCTG
AKR1C2b	ATAATGAGGAGCAGGTTGG	GCTCCAAAGCTTTGAAGTG
AKR1C3	TGCAACCAGGTAGAATGTC	TTTGTCTCGTTGAGATCCC
SRD5A1	TTTGAATACGTAACTGCAGC	GAGGTACCACTCATGATGC
SRD5A2	TTCTGCACTGGAAATGGAG	AATAAGAAGACACCCAAGCT
HSD3B1	GAATGTCAATGTGAAAGGTACC	CTACTGGTGTAGATGAAGACTG
HSD17B1	TCAATGACGTTTATTGCGC	GAGGTATTGGTAGAAGCGG
HSD17B3	TAGTCAAGATGACACAGCT	CTATCCCAGAAGAAATGTTCAG

References

1. Brawley, O.W., Prostate cancer and black men. Semin Urol Oncol, 1998. **16**(4): p. 184-6.
2. Abate-Shen, C. and M.M. Shen, Molecular genetics of prostate cancer. Genes Dev, 2000. **14**(19): p. 2410-34.
3. Austin, J.P., et al., Diminished survival of young blacks with adenocarcinoma of the prostate. Am J Clin Oncol, 1990. **13**(6): p. 465-9.
4. Koochekpour, S., et al., Androgen receptor mutations and polymorphisms in African American prostate cancer. Int J Biol Sci, 2014. **10**(6): p. 643-51.
5. Soos, G., et al., The prevalence of prostate carcinoma and its precursor in Hungary: an autopsy study. Eur Urol, 2005. **48**(5): p. 739-44.
6. Powell, I.J., et al., Outcome of African American men screened for prostate cancer: the Detroit Education and Early Detection Study. J Urol, 1997. **158**(1): p. 146-9.
7. Tewari, A., et al., Factors contributing to the racial differences in prostate cancer mortality. BJU Int, 2005. **96**(9): p. 1247-52.
8. Litman, H.J., et al., Serum androgen levels in black, Hispanic, and white men. J Clin Endocrinol Metab, 2006. **91**(11): p. 4326-34.
9. Reichardt, J.K., GEN GEN: the genomic genetic analysis of androgen-metabolic genes and prostate cancer as a paradigm for the dissection of complex phenotypes. Front Biosci, 1999. **4**: p. D596-600.
10. Ellis, L. and H. Nyborg, Racial/ethnic variations in male testosterone levels: a probable contributor to group differences in health. Steroids, 1992. **57**(2): p. 72-5.
11. Ross, R.K., et al., 5-alpha-reductase activity and risk of prostate cancer among Japanese and US white and black males. Lancet, 1992. **339**(8798): p. 887-9.
12. Huggins, C. and C.V. Hodges, Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. CA Cancer J Clin, 1972. **22**(4): p. 232-40.
13. Tucci, M., et al., Addition of Docetaxel to Androgen Deprivation Therapy for Patients with Hormone-sensitive Metastatic Prostate Cancer: A Systematic Review and Meta-analysis. Eur Urol, 2015.
14. Belanger, B., et al., Comparison of residual C-19 steroids in plasma and prostatic tissue of human, rat and guinea pig after castration: unique importance of extratesticular androgens in men. J Steroid Biochem, 1989. **32**(5): p. 695-8.
15. Forti, G., et al., Three-month treatment with a long-acting gonadotropin-releasing hormone agonist of patients with benign prostatic hyperplasia: effects on tissue androgen concentration, 5 alpha-reductase activity and androgen receptor content. J Clin Endocrinol Metab, 1989. **68**(2): p. 461-8.
16. Mohler, J.L., et al., The androgen axis in recurrent prostate cancer. Clin Cancer Res, 2004. **10**(2): p. 440-8.
17. Nishiyama, T., Y. Hashimoto, and K. Takahashi, The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. Clin Cancer Res, 2004. **10**(21): p. 7121-6.
18. Geller, J. and J. Albert, Effects of castration compared with total androgen blockade on tissue dihydrotestosterone (DHT) concentration in benign prostatic hyperplasia (BPH). Urol Res, 1987. **15**(3): p. 151-3.
19. Nishiyama, T., et al., The change in the dihydrotestosterone level in the prostate before and after androgen deprivation therapy in connection with prostate cancer aggressiveness using the Gleason score. J Urol, 2007. **178**(4 Pt 1): p. 1282-8; discussion 1288-9.
20. Wright, M.E., et al., Prospective study of adiposity and weight change in relation to prostate cancer incidence and mortality. Cancer, 2007. **109**(4): p. 675-84.

21. Planat-Benard, V., et al., Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation, 2004. **109**(5): p. 656-63.
22. Hessvik, N.P., et al., Profiling of microRNAs in exosomes released from PC-3 prostate cancer cells. Biochim Biophys Acta, 2012. **1819**(11-12): p. 1154-63.
23. Klopp, A.H., et al., Concise review: Dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? Stem Cells, 2011. **29**(1): p. 11-9.
24. Thery, C., L. Zitvogel, and S. Amigorena, Exosomes: composition, biogenesis and function. Nat Rev Immunol, 2002. **2**(8): p. 569-79.
25. Lakkaraju, A. and E. Rodriguez-Boulan, Itinerant exosomes: emerging roles in cell and tissue polarity. Trends Cell Biol, 2008. **18**(5): p. 199-209.
26. Hugel, B., et al., Membrane microparticles: two sides of the coin. Physiology (Bethesda), 2005. **20**: p. 22-7.
27. Skog, J., et al., Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol, 2008. **10**(12): p. 1470-6.
28. Valadi, H., et al., Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol, 2007. **9**(6): p. 654-9.
29. Abd Elmageed, Z.Y., et al., Neoplastic reprogramming of patient-derived adipose stem cells by prostate cancer cell-associated exosomes. Stem Cells, 2014. **32**(4): p. 983-97.
30. Mathur, A., et al., Subverting ER-stress towards apoptosis by nelfinavir and curcumin coexposure augments docetaxel efficacy in castration resistant prostate cancer cells. PLoS One, 2014. **9**(8): p. e103109.
31. Gubelmann, C., et al., GETPrime: a gene- or transcript-specific primer database for quantitative real-time PCR. Database (Oxford), 2011. **2011**: p. bar040.
32. YaoTang,Linbo Wang, Olga Goloubeva,Mohammad Afnan Khan, Bin Zhang,and Arif Hussain. Divergent effects of castration on prostate cancer in TRAMP mice: possible implications for therapy. Clin. Cancer Res., 14(10):2936-43, 2008.
33. Datta, A., et al., Selective targeting of FAK-Pyk2 axis by alpha-naphthoflavone abrogates doxorubicin resistance in breast cancer cells. Cancer Lett, 2015. **362**(1): p. 25-35.
34. Gregg, J.L., et al., Analysis of gene expression in prostate cancer epithelial and interstitial stromal cells using laser capture microdissection. BMC Cancer, 2010. **10**: p. 165.

Second Manuscript

Stem-cell based selective delivery of alpha keto reductases for therapeutic targeting of residual androgens in prostate cancer

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Abstract

Background: Castration-resistant prostate cancer (CRPC) is an advanced metastatic form of PC with poor survival rates of approximately 5 years of follow-up. Although initially effective, hormonal therapy is marked by progression to CRPC over a period of 18–20 months. Androgen deprivation therapy (ADT) is the mainstay treatment of metastatic PC and is often impeded by development of CRPC, especially among African Americans (AA). Importantly, large body of evidence indicate that in the setting of 'castrate' serum testosterone levels, prostatic androgen concentrations remain at approximately 10–25% of the levels found in untreated patients well within the range capable of mediating continued androgen-receptor (AR) signaling and gene expression in patients with CRPC. The *de novo* synthesis of androgens by prostate tumors provides a significant survival advantage leading to the outgrowth of 'castration-adapted' tumors. Currently, there are no drugs that selectively target and inhibit residual androgens in the tumor microenvironment. Our present research attempts to This study attempts to exploit selective delivery by normal donor adipose derived stem cells (nASCs) of α-hydroxysteroid dehydrogenase (α-HSD), an alpha keto reductase and testosterone and DHT inactivating enzyme, by tumor tropic normal ASCs to hydrolyze "*intracrine*" androgens into inactive metabolites and inhibit PC cell growth in vitro and in tumor-bearing mice.

Methods: A bicistronic IRES lentiviral vector expressing the 3α-HSD was constructed by subcloning alpha keto reductase gene AKR1C14 (α-HSD) in frame with IL2-SS into the pLVX-IRES-ZsGreen1 vector. The nASCs were enriched by their tumor-homing potential using a transendothelial well system. The enriched nASCs cells were transduced with either the control or the AKR1C14-expressiing lentivirus construct. The expression of transduced gene was confirmed by qRT-PCR and immunoblotting. The activity of transgene to catabolize androgens was assessed by EIA kit (Cayman). The antitumor activity of the nASC secreted transgene, compared to a hr-α-HSD, was assessed by its ability to degrade testosterone and inhibiting survival of the androgen-dependent LNCaP cells cultured in presence the hormone. Cell viability was assessed by MTT and LDH assay assays and induction of androgen-regulated genes (PSA) was monitored by reporter assay. Enriched GFP-expressing nASCs were examined for their tumor-homing and engraftment by systemic administration in mice bearing LNCaP tumors. The efficacy of enriched pASCs to deliver, secrete α-HSD, hydrolyze androgens and induce tumor regression was investigated in by systemic administration in mice bearing LNCaP tumors.

Results: A reduction in testosterone concentration was observed when LNCaP cells were treated with conditioned media (CM) from AKR1C14 transduced nASCs in concentration and NAD dependent manner. Like hr-α-HSD**,** LNCaP cells treated with the conditioned media (CM) of pLVX-3αHSD-IL2-SS-IRES-ZsGreen1 transduced nASCs showed higher cytotoxicity and reduced cell viability in in concentration and NAD dependent manner compared to control treated cells. Additionally, a decreased in PSA promoter activity was observed by luciferase assays in cells transfected with αHSD-expressing lentivirus construct compared to control construct. The enriched GFP-expressing AKR1C14 transduced nASCs successfully engrafted in tumors when administered in LNCaP tumor-bearing mice. A reduction in tumor volume, though not significant, was observed in mice administered with αHSD-expressing lentivirus construct compared to controls.

Conclusions: Our data demonstrates that selective delivery of a αHSD by tumor-tropic nASCs can effectively hydrolyze residual androgens and potentially inhibit growth of PC cells under "castrate"

conditions. To reduce residual androgens and achieve effective therapeutic response, further *in vivo* studies are required to optimize the dose and frequency of αHSD-expressing nASCs in tumor-bearing mice. The results suggest that αHSD-expressing nASCs can be incorporated as a mutli-modality hormone targeting therapy to reduce tumor burden in CRPC patients, especially among AA men.

Introduction

Androgen deprivation therapy (ADT) is still the mainstay in prostate cancer treatment since the hormone dependent nature of the disease was discovered [1]. ADT is primarily targeted towards bringing post-castration serum testosterone concentration below 20ng/dL. Many studies have demonstrated presence of residual androgens in tumor microenvironment even after castration [2]. It has been shown that although castration reduced the DHT levels to 1ng/g in most patients, adrenal blockade by ketoconazole is further able to decrease the prostatic DHT [3]. It was also reported that the combination of androgen synthesis inhibitors with castration therapy lowered the concentration of prostatic androgens below the level achieved by castration alone [4-9]. It can be inferred from these studies that castrate levels of circulating androgens allows testosterone to persist in the tumor micro environment. Androgen receptor signaling with DHT concentration (~1 nm, 0.5 to 1.0 ng/g) has been reported both in vitro and in vivo [4, 10-14]. Clinically the relevance of intra-tumoral androgens in progression of disease post castration is supported by survival benefit observed in patients undergoing adrenalectomy and /or hypophysectomy [15, 16]. More importantly, series of new studies show a strong indication that response and resistance to abiraterone is associated with tumoral evidence of upregulated androgen synthesis, clearly demonstrating the importance of intratumoral androgen metabolism in CRPC tumor survival [3, 12, 17, 18]. More importantly so, a series of studies strongly indicate that abiraterone resistance increases with increasing androgen synthesis [3, 12, 17-19]. This underlines the importance of intratumoral androgen synthesis in progression of CRPC tumor. AKR1C14, rat homolog of human AKR1C4, enzyme is potent DHT hydrolyzing enzyme [20-25], it has been reported that it is 30-50 times more effective in hydrolyzing DHT. In this study we investigate if normal tumor-tropic ASCs transduced to express AKR1C14 are effective in inhibiting growth of androgen-dependent PCa cells ex-vivo and in vivo. nASCs collected from donors were enriched for migration towards tumor cells. The tumor-tropic cells were then transduced this AKR1C14. The enzyme secreted by transduced showed the ability to hydrolyze DHT as reflected Enzyme Immune Assay and cell survival studies. Further, we could achieve a successful engraftment of transduced stem cells in SCID mice bearing

LNCaP tumors. Only a small reduction tumor size was observed in mice inoculated with AKR1C14 transduced stem cells compared to the control.

Material and Methods

Cell lines: nASCs were generously donated by Dr. Gimble's lab (Pennington Biomedical Research Center, Baton Rouge), LNCAP-SF were a generous gift from Dr. Kawai's lab (Kanazawa University, Japan). LNCaP cells were obtained from ATCC. All stem cells were maintained in DMEM-F12, 20% FBS, 1% Anti-Anti, LNCaP-SF cells were maintained in RPMI-1640 media with 10% charcoal stripped FBS,1% Anti-Anti and LNCaP cells were maintained in RPMI-1640 media with 10% FBS and 1% Anti-Anti

Transendothelial Migration of Tumor-Tropic ASCs

To ensure tumor-tropicity, nASCs and pASCs populations with high tropism towards PCa cells (LNCaP) were enriched using an in vitro trans-endothelial migration (TEM) system. Human bone marrow endothelial cells (hBMEC-1) (kindly provided by Dr. Graça D. Almeida-Porada, Univ. of Nevada, Reno, NV) was cultured onto Matrigel- coated membrane inserts (8 µm pore size) in 12-well plates to generate a confluent hBMEC-1 barrier in the apical chamber. The permeability of the microvessel barrier was checked with Evans blue dye by colorimetric assays. Prostate cancer cells CM was added to the basal chamber in 1:1 ratio with SVF growth media (DMEM-F12 supplemented with 10% exosome depleted FBS). ASCs (1 x 10^5) were added to apical chamber with hBMEC barrier and allowed to migrate towards the CM in the lower chamber for 48 hr. ASC isolates with tropism towards PC cells were harvested from basal chamber and propagated (passage <5), stored and used in subsequent experiments.

Lentivirus Vector Construction

We employed the strategy described previously (Applied Stem Cell, Inc.) to generate lentivirus expressing the pLVX-IL2SS- AKR1C14 -IRES-GFP gene product. A lentivirus expression vector, pLVX-IRES-ZsGreen1 (Clontech, Inc.) expresses two transgenes from a bicistronic mRNA transcript, allowing ZsGreen1 to be used as an indicator of transduction efficiency and a marker for selection by flow cytometry. The AKR1C14 (NM_138547) rat cDNA clone coding for 3α-hydroxysteroid dehydrogenase (Type I, 3α-HSD) was obtained from Origene. The IL2-SS (signal sequence) was synthesized (IDT, Inc.) to enable secretion of AKR1C14 enzyme by the recipient cells. Initially, the AKR1C14 gene was cloned in-frame with IL-2SS at the N-terminus in pCR-II plasmid using EcoRI and EcoRV restriction enzymes and

adaptors and the DNA insert was sequence verified. The IL2SS- AKR1C14 sequence was PCR amplified with *Spe*I and *Not*I anchored primers. The PCR product was subcloned in SpeI-NotI digested pLVX-IRES-ZsGreen1 plasmid to generate pLVX-IL-2SS-Akr1c14-IRES-GFP construct.

Transduction

5 x 10^4 nASCs/well were seeded in a 6 well plate and cultured overnight in regular stem cell media. Following morning, the wells were washed thrice with HBSS and the plate was put on ice. 400ul of Opti-Mem (Thermofisher Scientific, Rockford, IL) media containing 5ug/ml Polybrene from Millipore (Billerica, MA) was added to each well. The control GFP lentivirus and AKR1C14 lentivirus (2µl/well) was added to respective wells using pre chilled pipette tips. 24 hours later, the media was aspirated, wells washed with plain HBSS and regular stem cell media was added to the wells. The lentivirus uptake was visually established by examining GFP expression under Leica DMI3000B microscope (Leica Microsystems Inc., Buffalo Grove, Illinois) and immunoblotting in conditioned media as well qRT-PCR as described before [26] (data not shown). .

Preparation and microconcentration of nASC-AKR1C14 conditioned media (CM)

Cells were cultured in stem cell media for 36 hours, following which cells washed with HBSS thrice and plain DMEM-F12 was added the culture flask. The conditioned media was collected at 48hrs, centrifuged at 4000 RPM for 10 minutes 4°C to remove any cells and debris. The supernatant was sterile filtered using 0.2 micron filters from Millipore (Billerica, MA) following which, the filtered media was micro concentrated to 200 µL using Millipore 3 Kilo Dalton Molecular weight cut off filters. The concentrated media was quantitated using the BCA protein assay kit from Pierce (Thermofisher Scientific, Rockford, IL).

Western blotting:

To examine the expression of AKR1C14 in the CM of secreted enzyme immunoblotting was performed as described before [27]. The samples were diluted 1:1 in Lamelli buffer and boiled for 5 minutes at 95°C following which they were incubated on ice for two minutes to denature the proteins. 4-20 % gradient SDS PAGE gels obtained from Bio-Rad were used to separate the proteins at 100V. Proteins were then, transferred to a PVDF membrane using the trans-blot turbo transfer pack from Biorad (Hercules, CA) using Bio-Rad's transblot turbo apparatus at 15 mv for 10 minutes. Blocking buffer (Licor, Lincoln, NE) was used to block the membrane following the transfer. The membrane was then probed with desired primary and secondary antibody and imaged on Odyssey CLX (Licor, Lincoln, NE). The primary antibody raised against AKR1C14 was purchased from Genetex (Irvine, CA), Secondary antibody was obtained from Licor (Lincoln, NE).

Transfection

LNCaP-SF cells were transfected with AKR1C14 plasmid using Hi-perfect obtained from Qiagen (Venlo, Limburg) as per manufacturer's guidelines.

Cell viability Assay

To estimate cell viability by mitochondria were counted by estimating the conversion of yellow tertazoluim salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, St Louis, MO) to a purple colored formazan in living cells as described before [28]. Cell were plated in 96 well plate and allowed to adhere over night following the desired treatment the supernatant from the media was collected for LDH assay and the 10ul to of 5mg/ml stock (MTT) was added to each well containing fresh 100ul media. The cells were allowed to incubate for 3 hours following which the absorbance was measured at 540 nm using the µQuant plate reader from BioTek (Seattle, WA).

Cytotoxicity Assay

Supernatants from the cells that had undergone the treatment was used to determine the activity of Lactate dehydrogenase in the media using Cytotoxicity assay kit from Roche' (Basel Switzerland) as per manufacturer's guidelines. Lactate dehydrogenase is released in the media when the plasma membrane is lysed therefore the activity of LDH in the media was used a function of cytotoxicity in the cells.

ELISA

EIA ELISA kit was obtained from Cayman (Ann Arbor, MI).Testosterone concentration was estimated according to manufacturer's guidelines.

Immunofluorescence analysis

Tumors were resected from euthanized mice as per approved protocol. Tumor sections were snap-frozen or paraffin-embedded for further analysis. IHC was performed as described before to examine the engraftment of stem cells into the tumor [29] Tissue sections were fixed on glass slide, deparaffinization and antigen retrieval was performed at histology core facility in the Department of Pathology, Tulane University School of Medicine. After the antigen retrieval the samples were blocked with 5% of goat serum in PBS and 0.2% Triton-X for 1 hour. Blocked samples were washed twice with 0.2% Triton X- 100 in PBS, then incubated (4°C, 12–16 h) with primary antibodies against GFP obtained from Genetex (Ann Arbour, MI). Tissue sections were then incubated for 1hr at room temperature in PBS 0.2% Triton X- 100 solution containing secondary antibodies conjugated with Alex Flour 488, 594, or 647- conjugated IgGs; (Molecular Probes, Invitrogen, Carlsbad, CA). Slides were mounted with mounting medium with DAPI to stain the nuclei (Vectashield, Burlingame, CA). Images were acquired using Leica DMI3000B microscope (Leica Microsystems Inc., Buffalo Grove, Illinois).

Statistical Analysis

All experiments were conducted thrice and data generated was analyzed in SAS 9.4 software. MTTs were analyzed in repeated measure ANOVA, ELISA samples were quantitated by four way logistic regression and quantitated samples were compared using ANOVA.

Results

Enrichment of tumor-tropic nASCs

The nASCs with tropism towards LNCaP cells were enriched *in vitro* by a trans-endothelial migration (TEM) system encompassing human bone marrow endothelial cells (hBMEC-1) barrier in the apical chamber. The ASCs were allowed to migrate for 48 hr from the apical chamber towards the conditioned medium (CM) of LNCaP cells in the lower chamber. About 7-10% efficiently migrated to the lower chamber. Cells were harvested, propagated in cultured and stored (passage <5).

The transduced tumor tropic nASCs secrete 3αalpha HSD (Type 1, 3αHSD) enzyme

A bicistronic IRES lentiviral vector expressing the 3α-HSD was constructed by subcloning alpha keto reductase gene AKR1C4 (α-HSD) in frame with IL2-SS into *Spe*I and *Not*I sites in the pLVX-IRES-ZsGreen1 plasmid and sequence verified (Figure 1A). The IL2 signaling sequence (IL2-SS) facilitates secretion of the transgene by the recipient cells. The control (EF1-alpha GFP) and recombinant plasmids (pLVX-3αHSD-IL2-SS-IRES-ZsGreen1) were used to generate lentivirus vectors.

To assess transduction efficiency and ability of nASCs to secrete Type 1, 3αHSD enzyme, the enriched nASCs were transduced with the control expression vector or the recombinant AKR1C14-expressing expression plasmid and cells were examined for expression of GFP 24 hours following transduction. As shown in Figure 1B, the transduction efficiency of the nASCs was >95%. Additionally, the conditioned medium (CM) of the transduced cells was examined for secretion of Type 1, 3αHSD by immunoblot analysis. Figure 1C depicts release of AKR1C14-IL2-SS protein. The bands appears to be slightly larger compared to recombinant AKR1C14 protein (Figure 1C).

NASC-secreted 3αHSD enzyme degrades testosterone and inhibits its activity in PC cells

To verify that the nASC-secreted 3αHSD depletes exogenous dihydrotestosterone (DHT) in LNCaP cultures, ELISA was performed in LNCaP cells maintained in hormone-derivation conditions and

supplemented with 10 nM DHT in presence or absence of 2.5mM NAD and different concentrations of CM of 3αHSD-expressing nASCs for 48 hrs. NAD is required for activation of 3αHSD enzyme. As shown in Figure 2, significantly lower DHT concentration ($p<0.001$) was observed in LNCaP cells treated with recombinant AKR1C14 protein or CM from AKR1C14 transduced nASCs compared to treatment with CM from control cells. The results suggest that the 3αHSD enzyme secreted by transduced nASCs is active and capable of catabolizing DHT.

To confirm that enzyme was active, dual luciferase assay was performed to examine if the enzyme inhibits the promoter activity of prostate specific antigen (PSA) in presence of androgens. LNCaP cells were transfected with psPSA-luciferase plasmid, a reporter plasmid that drives expression of luciferase gene under the control of two truncated PSA promoter regions encompassing ARE. The cells were then cultured in charcoal-stripped media supplemented with testosterone (10 nM) in presence or absence of various concentrations (0.05-0.25 µg/µL) of the nASC CM and NAD. As shown in Figure 3, a decrease in the PSA promoter activity was observed in the LNCaP cells treated with CM of AKR1C14 transduced nASCs or AKR1C14 recombinant protein in presence, but not in the absence, of NAD ($p<0.05$).

Treatment with AKR1C14-transduced nASCs CM reduces cell survival and induces cytotoxicity in androgen dependent LNCaP cells

Androgen dependent LNCaP cells cultured in complete medium (10% FBS) were treated with varying concentration of CM of AKR1C14 transduced stem cells or the control stem cells or the recombinant protein in the presence or absence of NAD. Reduced cell survival (Fig. 4) and increased cytotoxicity (Fig. 5) were observed in cells treated with either the recombinant AKR1C14or CM of AKR1C14-IL2SS-transduced stem cells in a concentration dependent manner in the presence, but not in absence, of NAD. Since the enzyme requires NAD, it appears that the secreted AKR1C14 actively catabolized and depleted androgen in the medium, and subsequently reduced cell survival of the androgen-dependent LNCaP cells.

AKR1C14-transduced nASCs home to and regress LNCaP tumor growth in nude mice

Next, we examined the tumor-homing potential of the enriched nASCs and transduced with AKR1C14-IL2SS-GFP lentivirus expression vector, and further examined their ability to regress tumor growth *in vivo*. Gonad-intact athymic nu/nu male (5-6 weeks old) (Taconic) were subcutaneously injected with LNCaP (4×10^6) cells suspended in 50 μL of serum-free RPMI-1640 medium and Matrigel (BD Bioscience, MD) (1:1). Upon tumor formation (~ 6 weeks), the animals were injected with the enriched pLV-AKR1C14-IL2SS-GFP transduced nASCs (1×10^6) via tail vein once every week for 4 weeks. As shown in Figure 6A, the enriched nASCs effectively engrafted in the LNCaP tumors, as evidenced by GFP expression. Figure 6B depicts the expression of AKR1C14 protein (Texas Red color) in the LNCaP tumors. At the end of 4 weeks of treatment, the tumor volume was measured and recorded using the formula [$0.5 \times L \times (W)^2$][30]. As shown in Figure 6C, a reduction of LNCaP tumors was observed in mice treated with AKR1C14-expressing nASCs compared to baseline tumor volumes.

Discussion

To explore the possibility to reduce the tumor burden by delivery of androgen hydrolyzing enzyme mouse derived AKR1C14 for hydrolyzing circulating androgens and potentially reducing tumor burden. We transduced tumor tropic stem cells derived from non-diseased subjects with AKRC14 enzyme. The examined the expression of the enzyme in the CM of the stem cells using immunoblot. Once the expression was verified by immunoblot we wanted to examine if the secreted protein was indeed an active enzyme we performed a few experiments. First, we examined the ability of CM derived from AKR1C14 transduced stem cells to induce cytotoxicity and reduce cell survival in androgen dependent LNCaP cells. It was seen both recombinant AKR1C14 and AKR1C14 transduced stem cells exhibited cytotoxic effect in a NAD dependent manner. This is a promising indicator of an active enzyme, the same effect was also observed when DHT concentration and androgen receptor promoter activity was examined in the cells treated with recombinant AKR1C14, CM derived from AKR1C14 transduced stem cells compared to the cells treated with CM derived control plasmid. The results obtained from our study indicate that the potential of AKR1C14 transduced tumor tropic stem cells to reduce tumor burden by hydrolyzing the circulating androgens in the tumor microenvironment.

Higher circulating concentration of E2 has been reported in prostate cancer patients that have undergone radical prostatectomy. Previously reported interaction between androgen receptor and estrogen receptor make the circulating concentration of E2 more relevant to prostate cancer [31]. Our data showed that treatment with E2 for 24 hours corresponded with increased DHT concentration, suggesting that circulating E2 could be driving DHT synthesis. Concentration of DHT in LNCaP-SF cells transfected with AKR1C14 showed a significantly lower concentration of DHT at 24 hours compared to the control confirming the ability of secreted AKR1C14 to catabolize DHT in castration adapted state ex vivo. To examine the ability of AKR1C14 secreted by tumor tropic stem cells to catabolize circulating androgens in vivo and reduce tumor burden, we injected AKR1C14 transduced stem cells in five mice bearing LNCaP tumors. The reduction in tumor volume after inoculation with AKR1C14 transduced stem cells showed no

significant difference, it is possible androgen deprivation therapy in combination with stem cell treatment could have achieved synergy and shown a significant regression in tumor.

Conclusions

It can be concluded from our study that AKR1C14 transduced tumor tropic stem cells can secrete an active 3αHSD. The secreted enzyme us capable of catabolizing testosterone ex-vivo as demonstrated by PSPSA LUC reporter assay, cell survival studies and DHT ELISA. The transduced tumor tropic stem cells were successfully engrafted on the tumor size although no significant remission in tumor volume was observed. It is possible that a combination of bicalutamide with tumor tropic stem cells transduced with AKR1C14 could have enabled a significant reduction in tumor volume.

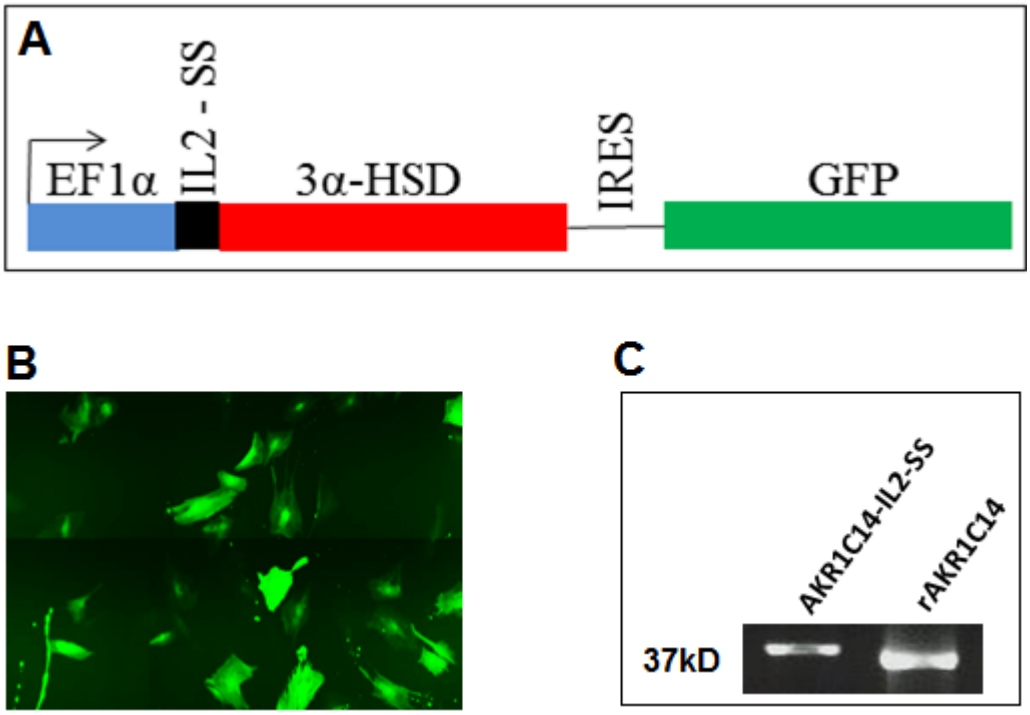

Figure 1. Construction of the recombinant lentivirus expression vector and expression and release of the transgene by nASCs. **(A)** a recombinant bicistronic pLVX-IRES-ZsGreen1 expression vector expression of 3αHSD (AKR1C14) and IL2 signal sequence (IL2-SS) to enable secretion of the transgene by the transduced nASCs. Details of the subcloning strategy are described in Materials and Methods section. (**B**) a representative image demonstrating GFP expression, reflecting the transduction efficiency of the expression vector in the enriched tumor- tropic stems nASCs. **(C)** The conditioned media (CM) of tumor tropic nASCs stem cells transduced with the recombinant expression vector was examined for secretion of 3αHSD enzyme. Shown is an immunoblot analysis of the CM depicting expression and release of AKR1C14-IL2-SS protein. The recombinant AKR1C14 protein was used as control.

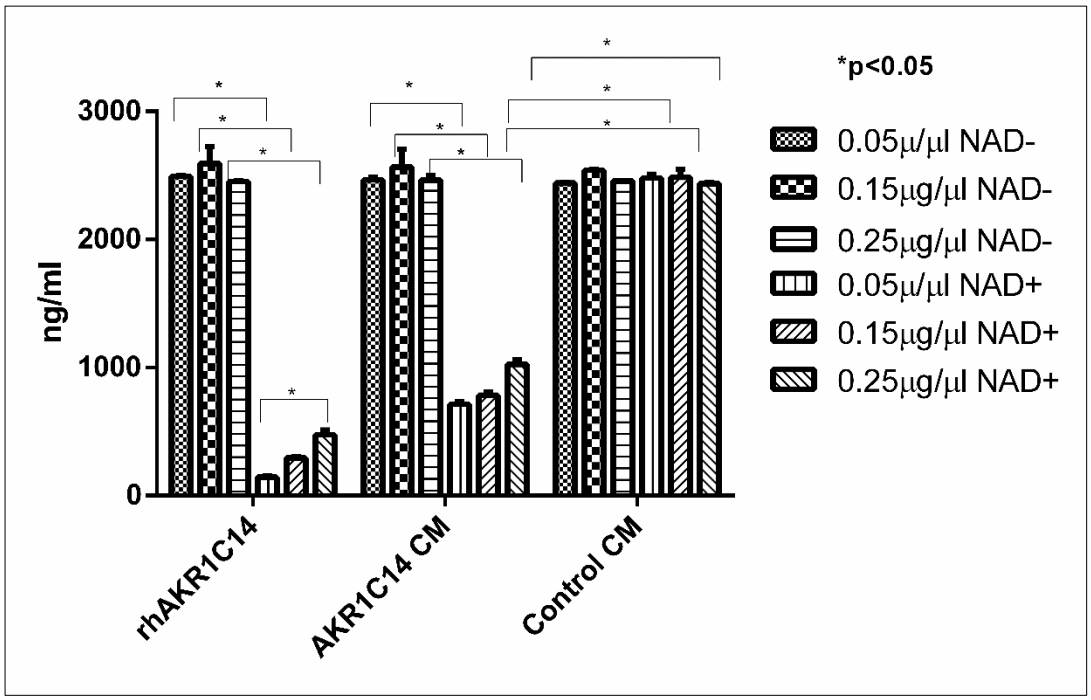

Figure 2. nASC-secreted 3αHSD depletes exogenous testosterone in LNCaP cultures. ELISA performed in LNCaP cells supplemented with 10 nM DHT with and without 2.5mM NAD and different concentrations of CM of 3αHSD-expressing nASCs for 48 hrs. Significantly lower DHT concentration was observed in LNCaP cells treated with recombinant AKR1C14 and CM from AKR1C14 transduced stem cells compared to treatment with CM from control cells. * denotes significance at $p<0.001$.

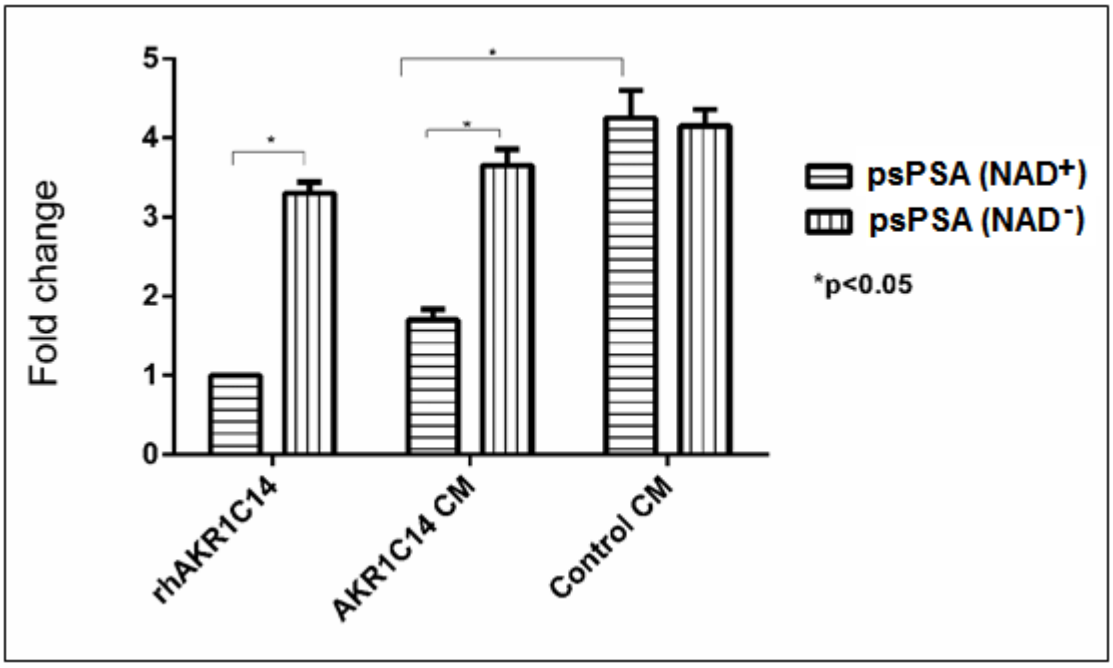

Figure 3. nASC-secreted 3αHSD suppresses expression of androgen-regulated PSA in LNCaP cells. A psPSA dual luciferase reporter assay (Promega) was performed in the androgen- dependent LNCaP cells. The cells were cultured in charcoal-stripped FBS supplemented with exogenous DHT (10 nM) and subsequently treated with CM of nASCs transduced with 3αHSD-expressing construct, control CM or the human recombinant AKR1C4 for 48 hours in the presence and absence of 2.5mM NAD, required for activation of 3αHSD. Reduced promoter activity was observed in recombinant AKR1C14 and CM in presence of NAD. * denotes significance at $p<0.05$)

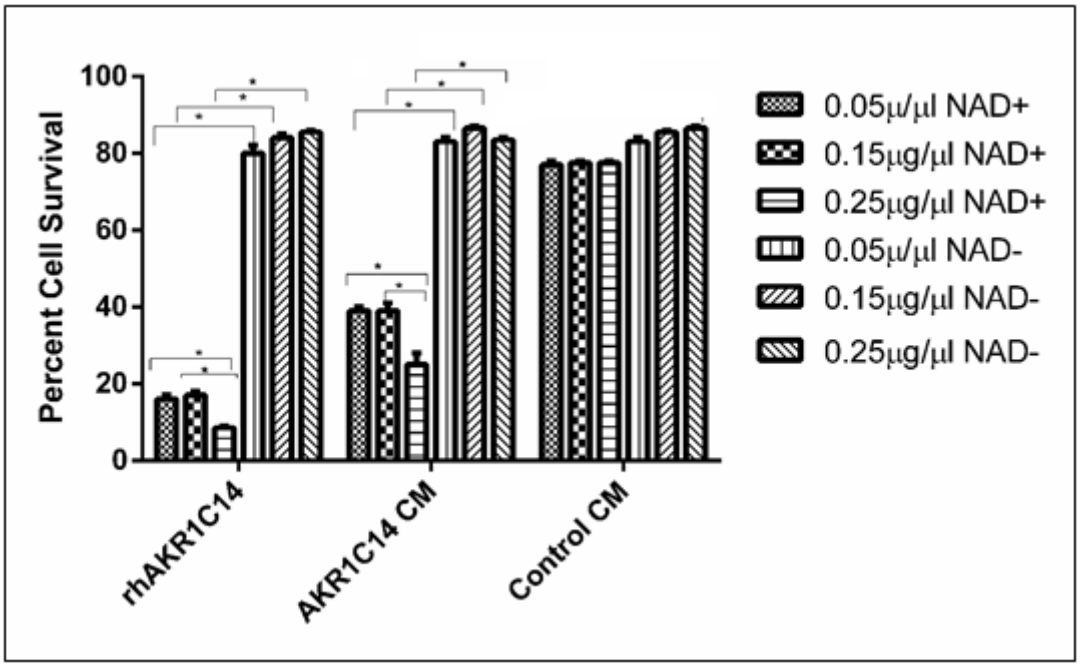

Figure 4. nASC-secreted 3αHSD reduced survival of the androgen-dependent LNCaP cells. LNCaP cells were cultured in a 96-well plates in charcoal-stripped FBS supplemented with exogenous DHT (10 nM) and subsequently treated with varying concentrations of CM of nASCs transduced with 3αHSD-expressing construct, control CM or the human recombinant AKR1C4 for 48 hours in the presence and absence of 2.5mM NAD, required for activation of 3αHSD enzyme. Cell cultured in NAD-containing media showed reduced cell survival upon treatment with rh3αHSD or CM derived from nASCs transduced with 3αHSD-expressing construct in a concentration dependent manner, when examined by MTT assay. * denotes significance at $p<0.05$.

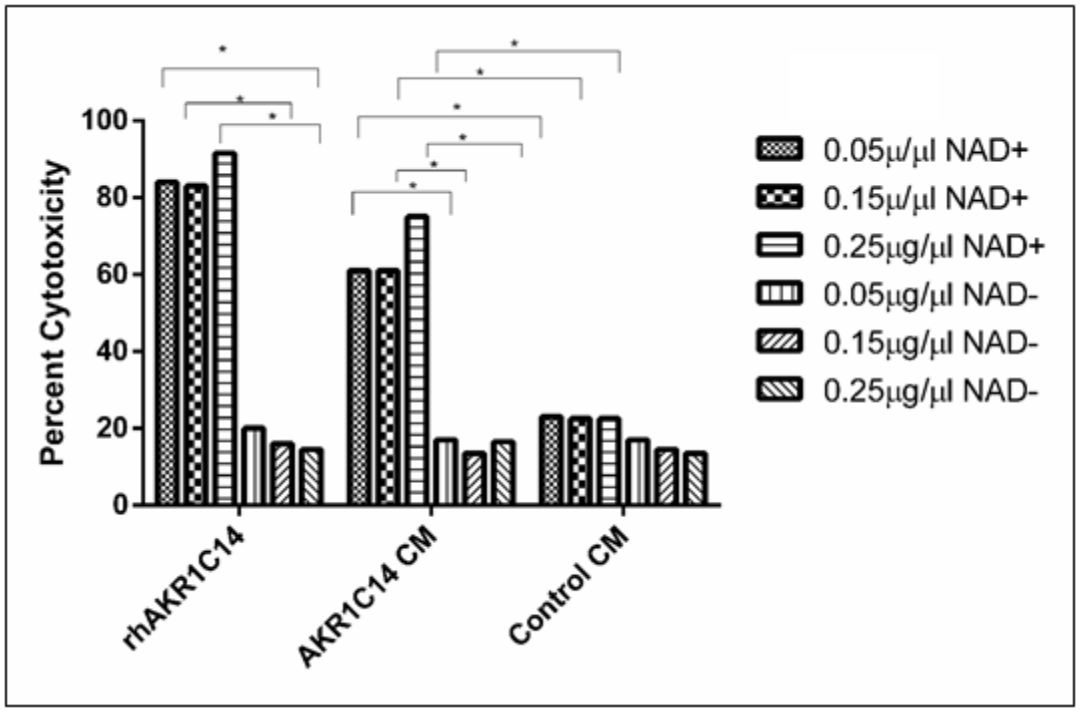

Figure 5. nASC-secreted 3αHSD increases cytotoxicity in the androgen-dependent LNCaP cells. LNCaP cells were cultured in a 96-well plates in charcoal-stripped FBS supplemented with exogenous DHT (10 nM) and subsequently treated with varying concentrations of CM of nASCs transduced with 3αHSD-expressing construct, control CM or the human recombinant AKR1C4 for 48 hours in the presence and absence of 2.5mM NAD, required for activation of 3αHSD enzyme. Cell cultured in NAD-containing media showed increased cytotoxicity upon treatment with rh3αHSD or CM derived from nASCs transduced with 3αHSD-expressing construct in a concentration dependent manner, when examined by LDH assay. * denotes significance at $p<0.05$.

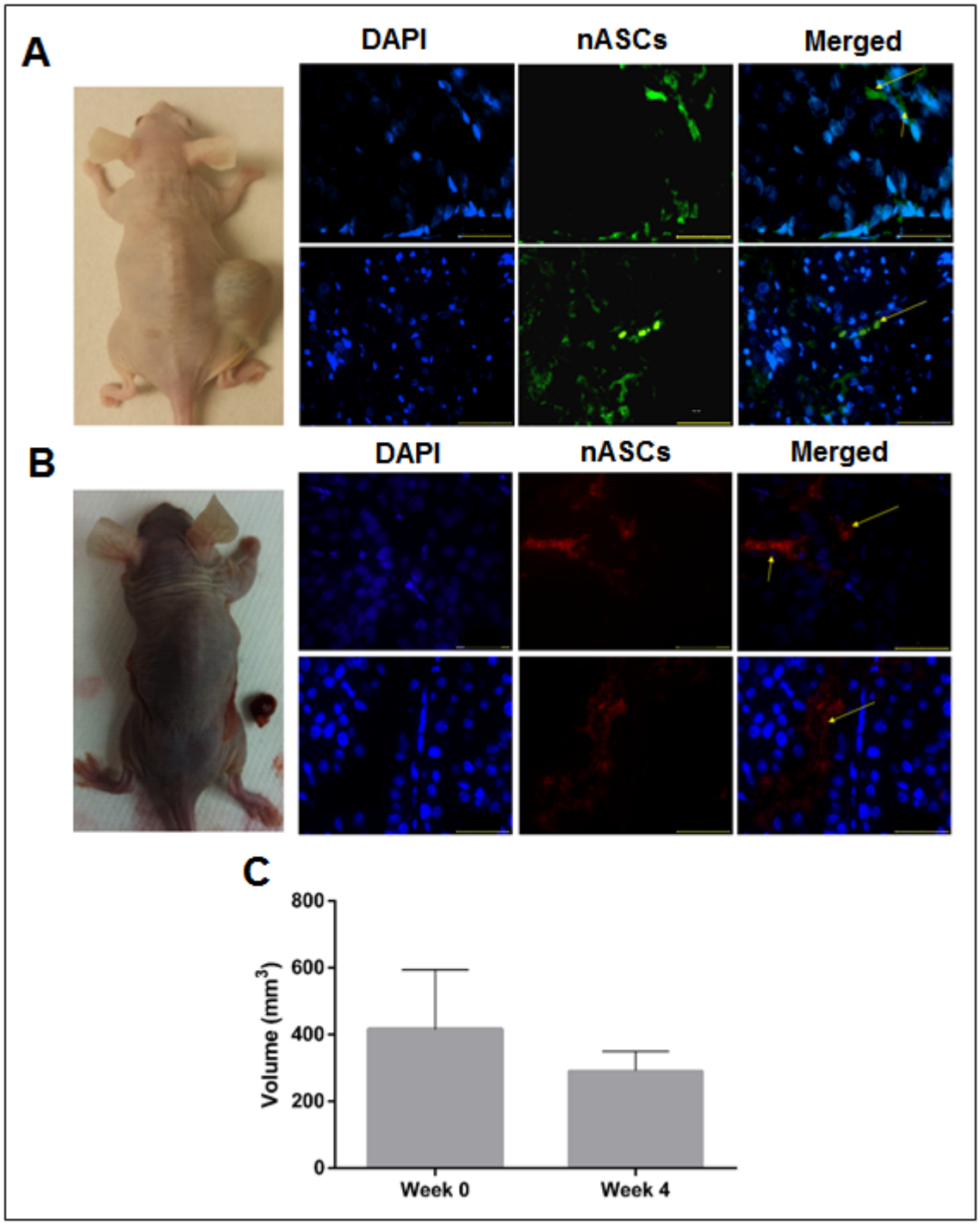
A
DAPI
nASCs
Merged
B
DAPI
nASCs
Merged
C
800
600
400
200
0
Volume (mm3)
Week 0
Week 4

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Figure 6. AKR1C14-transduced nASCs homing to LNCaP tumor *in vivo* and induces tumor regression. A, the tropism of enriched nASCs towards LNCaP tumors was examined in mice bearing LNCaP tumors (n=5; *left panel*). The tropism of enriched nASCs towards LNCaP tumors was confirmed by administering GFP transduced stem cells ($1x10^6$) by i.v. and their engraftment by was evaluated by antiGFP antibody as shown in two representative IHC images (*center and right panels*) of the tumor sections. B, mice were inoculated s.c with 4×10^6 LNCaP cells and tumor size was monitored every week. Upon tumor formation ($100\ mm^3$) 1×10^6 GFP- AKR1C14-expressing nASCs were injected weekly i.v route for 4 weeks in each mouse using previously optimized conditions. Texas Red secondary antibody was used to detect expression of AKR1C14 transduced stem cells. Nuclei are stained with blue color (DAPI). C, tumor volumes were measured using formula $0.5xLxW^2$. Mean tumor size was compared between the initial tumor volume and after treatment with AKR1C14-expressing nASCs using *paired t test* ($P<0.11$)

Acknowledgements

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References:

1. Huggins, C. and C.V. Hodges, *Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941.* J Urol, 2002. **168**(1): p. 9-12.
2. Mostaghel, E.A. and P.S. Nelson, *Intracrine androgen metabolism in prostate cancer progression: mechanisms of castration resistance and therapeutic implications.* Best Pract Res Clin Endocrinol Metab, 2008. **22**(2): p. 243-58.
3. Mostaghel, E.A., *Steroid hormone synthetic pathways in prostate cancer.* Transl Androl Urol, 2013. **2**(3): p. 212-227.
4. Geller, J. and J. Albert, *Effects of castration compared with total androgen blockade on tissue dihydrotestosterone (DHT) concentration in benign prostatic hyperplasia (BPH).* Urol Res, 1987. **15**(3): p. 151-3.
5. Geller, J., et al., *DHT concentrations in human prostate cancer tissue.* J Clin Endocrinol Metab, 1978. **46**(3): p. 440-4.
6. Geller, J., et al., *Medical castration of males with megestrol acetate and small doses of diethylstilbestrol.* J Clin Endocrinol Metab, 1981. **52**(3): p. 576-80.
7. Geller, J., et al., *Relationship between human prostatic epithelial cell protein synthesis and tissue dihydrotestosterone level.* Clin Endocrinol (Oxf), 1987. **26**(2): p. 155-61.
8. Liu, J., J. Albert, and J. Geller, *Effects of androgen blockade with ketoconazole and megestrol acetate on human prostatic protein patterns.* Prostate, 1986. **9**(2): p. 199-205.
9. Liu, J., et al., *Acute effects of testicular and adrenal cortical blockade on protein synthesis and dihydrotestosterone content of human prostate tissue.* J Clin Endocrinol Metab, 1985. **61**(1): p. 129-33.
10. Culig, Z., et al., *Switch from antagonist to agonist of the androgen receptor bicalutamide is associated with prostate tumour progression in a new model system.* Br J Cancer, 1999. **81**(2): p. 242-51.
11. Gregory, C.W., et al., *Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes.* Cancer Res, 1998. **58**(24): p. 5718-24.
12. Gregory, C.W., et al., *Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen.* Cancer Res, 2001. **61**(7): p. 2892-8.
13. Mohler, J.L., et al., *The androgen axis in recurrent prostate cancer.* Clin Cancer Res, 2004. **10**(2): p. 440-8.
14. Mohler, J.L., et al., *Identification of differentially expressed genes associated with androgen-independent growth of prostate cancer.* Prostate, 2002. **51**(4): p. 247-55.
15. Greenberg, E., *Endocrine therapy in the management of prostatic cancer.* Clin Endocrinol Metab, 1980. **9**(2): p. 369-81.
16. Robinson, M.R., R.J. Shearer, and J.D. Fergusson, *Adrenal suppression in the treatment of carcinoma of the prostate.* Br J Urol, 1974. **46**(5): p. 555-9.
17. Cai, L., et al., *Suppression of hepatocyte growth factor production impairs the ability of adipose-derived stem cells to promote ischemic tissue revascularization.* Stem Cells, 2007. **25**(12): p. 3234-43.
18. van Weerden, W.M., et al., *Effects of low testosterone levels and of adrenal androgens on growth of prostate tumor models in nude mice.* J Steroid Biochem Mol Biol, 1990. **37**(6): p. 903-7.
19. Miyamoto, H., et al., *Delta5-androstenediol is a natural hormone with androgenic activity in*

human prostate cancer cells. Proc Natl Acad Sci U S A, 1998. **95**(19): p. 11083-8.
20. Rizner, T.L. and T.M. Penning, *Role of aldo-keto reductase family 1 (AKR1) enzymes in human steroid metabolism.* Steroids, 2014. **79**: p. 49-63.
21. Jin, Y., et al., *Human cytosolic hydroxysteroid dehydrogenases of the aldo-ketoreductase superfamily catalyze reduction of conjugated steroids: implications for phase I and phase II steroid hormone metabolism.* J Biol Chem, 2009. **284**(15): p. 10013-22.
22. Steckelbroeck, S., et al., *Human cytosolic 3alpha-hydroxysteroid dehydrogenases of the aldo-keto reductase superfamily display significant 3beta-hydroxysteroid dehydrogenase activity: implications for steroid hormone metabolism and action.* J Biol Chem, 2004. **279**(11): p. 10784-95.
23. Penning, T.M., et al., *Structure-function relationships in 3alpha-hydroxysteroid dehydrogenases: a comparison of the rat and human isoforms.* J Steroid Biochem Mol Biol, 2003. **85**(2-5): p. 247-55.
24. Penning, T.M., et al., *Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones.* Biochem J, 2000. **351**(Pt 1): p. 67-77.
25. Nacusi, L.P. and D.J. Tindall, *Targeting 5alpha-reductase for prostate cancer prevention and treatment.* Nat Rev Urol, 2011. **8**(7): p. 378-84.
26. Miller, S., et al., *Genetic and epigenetic alterations of steroidogenic factor1 in ovarian tumors.* Int J Oncol, 2013. **42**(2): p. 627-34.
27. Abd Elmageed, Z.Y., et al., *Neoplastic reprogramming of patient-derived adipose stem cells by prostate cancer cell-associated exosomes.* Stem Cells, 2014. **32**(4): p. 983-97.
28. Mathur, A., et al., *Subverting ER-stress towards apoptosis by nelfinavir and curcumin coexposure augments docetaxel efficacy in castration resistant prostate cancer cells.* PLoS One, 2014. **9**(8): p. e103109.
29. Datta, A., et al., *Selective targeting of FAK-Pyk2 axis by alpha-naphthoflavone abrogates doxorubicin resistance in breast cancer cells.* Cancer Lett, 2015. **362**(1): p. 25-35.
30. Jensen, M.M., et al., *Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper.* BMC Med Imaging, 2008. **8**: p. 16.
31. Abd Elmageed, Z.Y., et al., *High circulating estrogens and selective expression of ERbeta in prostate tumors of Americans: implications for racial disparity of prostate cancer.* Carcinogenesis, 2013. **34**(9): p. 2017-23.

What opportunities for training and professional development has the project provided?

Part of a graduate student research work was based on studies related to this project. The student successfully defended his Ph.D. thesis in November, 2015.

How were the results disseminated to communities of interest?

The results of the studies were presented at the AACR annual conference in 2015. Our findings attracted a surge of interest by scientists and pharmaceutical companies. The data was also presented at Tulane University through (a) Tulane Annual Research Days, (b) Tulane Cancer Center Annual Retreat, and (C) Graduate Program in Biomedical Sciences Annual Retreat.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

IMPACT: *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

- **What was the impact on the development of the principal discipline(s) of the project?**

The following are the important findings that may impact understanding of prostate cancer progressions and its therapeutic implications

a) Our findings describe a new mechanism for the *de novo* synthesis of androgens in the tumor microenviroments by prostate cancer patients' derived tumor-tropic mesenchymal stem cells.

b) A novel finding is the potential role the PC cell derived exosomes in mediating the *de novo* synthesis of androgens through trafficking of genetic materials and/or proteins from cancer cells into the tumor-recruited stem cells.

c) Importantly, we also demonstrated that the tumor-tropic stem cells, especially those procured from African Americans (AA), can augment growth of prostace cancer cells under hormone-deprivation conditions, both *in vitro* and *in vivo*. Our results support a new mechanism for tumor-tropic ASCs in supporting clonal expansion of metastatic prostate tumors, especially among AA patients with castration-resistant prostate cancer (CRPC).

d) Our data demonstrates that selective delivery of a αHSD by tumor-tropic normal stem cells can effectively hydrolyze residual androgens and potentially inhibit growth of PC cells under "castrate" conditions.

e) Importantly, our results suggest that αHSD-expressing normal stem cells can be incorporated as a mutli-modality hormone targeting therapy to reduce tumor burden in CRPC patients, especially among AA men.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Our findings are likely to make an impact on development of new therapeutic strategies for advance and CRPC, especially among AA men.

What was the impact on society beyond science and technology?

Nothing to Report

CHANGES/PROBLEMS:

Although a trend in reduction of tumor sizes was observed in our therapeutic approach using engineered 3αHSC-expressing stem cells (second paper), further *in vivo* studies are required to optimize the dose and frequency of αHSD-expressing stem cells in tumor-bearing mice to reduce residual androgens and achieve an effective therapeutic response.

PRODUCTS:

- **Journal publications.**

- The two attached manuscripts (above) will be submitted for publication over the next three weeks. Acknowledgement of federal support is included in both manuscripts.

- Yang Y, Jia D^2, Kim H, Abd Elmageed ZY, Datta A, Davis R, Srivastav S, Moroz K, Crawford B, Moparty K, Thomas R, Hudson RS, Ambs S, Abdel-Mageed AB. Dysregulation of microRNA-212 Promotes Castration Resistance via hnRNPH1-Mediated Regulation of AR and AR-V7: Implications for Racial Disparity of Prostate Cancer. Clin Cancer Res. 2015 Nov 9. [Epub ahead of print]

- Silberstein JL, Feibus AH, Maddox MM, Abdel-Mageed AB, Moparty K, Thomas R, Sartor O. Active surveillance of prostate cancer in African American men. Urology. 2014 Dec;84(6):1255-61.

- **Books or other non-periodical, one-time publications.**

Nothing to Report

- **Other publications, conference papers, and presentations.**

The study findings were presented at the AACR annual meeting.

http://cancerres.aacrjournals.org/content/75/15_Supplement/3537.abstract

(*see copy of the AACR Proceedings on the next page*).

- **Website(s) or other Internet site(s)**

  - The research data was presented at Tulane University Health Research Days https://tulane.edu/asvpr/upload/ALL-ABSTRACTS-03-21-14.pdf

  - The research data was also disseminated through Tulane Cancer Center Annual Retreat

    http://www.louisianacancercenter.org/wp-content/uploads/2013/01/2015-Retreat-Booklet-Final-Version.pdf

Technologies or techniques

The technique designing a lentivirus construct for the expression and release of the 3αHSD by nASCs is novel.

Inventions, patent applications, and/or licenses

The Tulane Office of Technology transfer advised us not to proceed with patent application for the above technique because the method details were inadvertently disseminated on the intent through one of our DoD annual reports.

Other Products

The 3αHSD-expressing lentivirus construct is available for additional preclinical therapeutic evaluation/optimization in animal models of advance prostate cancer

2. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Asim B Abdel-Mageed, DVM, MS, Ph.D. PI (No change)

Debasis Mondal, Ph.D., Co-PI (no change)

Bruce A Bunnell, Ph.D., Co-investigator (no change)

Raju Thomas, MD, Co-investigator (no change)

Zakaria Abdel-Mageed , Ph.D., (postdoctoral Fellow), no change

Yijung Yang , Ph.D., (postdoctoral Fellow, no change

Manish Ranjan, BSc., no change

:

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report

- **What other organizations were involved as partners?**

Nothing to Report

3. **SPECIAL REPORTING REQUIREMENTS**

Nothing to Report